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Differential activation of dendritic cell subsets by *Schistosoma mansoni*

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DECLARATION

I, the undersigned, hereby declare that the contents of this thesis have been composed by myself and that the work described herein is entirely my own unless acknowledged otherwise.

Lauren Michelle Webb

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EXPERIMENTAL CONTRIBUTIONS

The FLDC project was developed in collaboration with Dr Rachel Lundie. Rachel performed the FLDC sorting and CD205-deficient FLDC experiments in chapter 3, as well as the gene expression analysis of *S. mansoni* infected livers in chapter 5.

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LAY SUMMARY

Dendritic cells (DCs) play a critical role in the immune system, recognising invading pathogens and activating other cells that can fight off the attack. One such invader is the parasitic worm *Schistosoma mansoni*, which afflicts millions of people in the tropics. Adult worms, once inside the host, reside within the blood system, where female worms lay hundreds of eggs a day. Surprisingly, the adult worms remain largely invisible to the immune system – it is the eggs that activate a strong response. Parasite eggs must pass through the wall of the intestine to escape the host, but very few make it that far, with the majority being swept by blood flow into the liver or getting stuck in intestinal tissue. This causes lots of damage to the intestine and the liver. In an attempt to control this damage, immune cells form a wall around the eggs, protecting other cells from the secretions they release. DCs are crucial to stimulating the response against *S. mansoni* eggs. In order to understand how DCs can do this, we have studied how they respond to molecules that make up the parasite eggs, and have identified signals that are essential for optimal DC function. We have also investigated whether these signals are required when DCs interact with and activate other immune cells. However, there is not just one type of DC, but many different subsets within the different tissues in the body. As well as using cells grown in culture, we have studied the responses of DC subsets in the spleen, lymph nodes and the intestine following injection of parasite eggs, but also the characteristics of different DCs during active infection. A greater understanding of these cells and the different roles they play in immune activation against *S. mansoni* is essential for the development of therapies that could help to augment or alter the immune response during worm infection. Not only that, this knowledge also helps us to understand what is happening when the immune system goes wrong in allergic disease and asthma.

ABSTRACT

Dendritic cells (DCs) play an essential role in bridging the innate and adaptive immune system, activating T cell responses against invading pathogens. It has been shown definitively that DCs fulfil the vital role of activating Th2 responses in the liver and spleen during infection with the parasitic helminth *Schistosoma mansoni*. However, DCs are an incredibly heterogeneous cell type, with diverse subsets displaying different phenotypes and functions in specific tissues in the body. Moreover, relatively little is known about how DCs become activated and stimulate T cells in response to Th2-associated parasitic helminths. This thesis addresses how distinct DC subsets function in response to schistosomes, both *in vitro* and *in vivo*.

The primary DC differentiation factor, Flt3-L, generates DC subsets *in vitro* that are analogous to the subsets resident in the lymphoid organs in the steady-state: $CD24^+$ conventional DCs (cDCs, $CD8\alpha^+$ equivalents), $CD11b^+$ cDCs and plasmacytoid DCs (pDCs). These different DC subsets displayed distinct responses to the strongly Th2-polarising soluble egg antigens (SEA) from *S. mansoni*. pDCs are unlikely to play a role in priming the Th2 response against SEA, although pDCs upregulated surface expression of MHC II and co-stimulatory molecules, these markers were expressed only at very low-levels, and pDCs failed to migrate to the draining lymph node (dLN) following adoptive transfer. In contrast, cDCs migrated efficiently to the T cell zone of the dLN. $CD11b^+$ and $CD24^+$ cDCs also significantly upregulated expression of the surface markers associated with T cell priming in response to SEA, however, this was a muted surface phenotype when compared to the classical activation elicited by a bacterial stimulus.

The DC subsets produced very little cytokine in response to SEA stimulation, with the exception of Type I Interferons (IFN-I), which were uniquely secreted by $CD24^+$ cDCs. The Toll-like receptor (TLR) adaptor proteins, TRIF and MyD88, were revealed to have contrasting roles in the control of SEA-specific IFN-I production. TRIF was essential for this response, whilst MyD88 acted as a negative regulator. TLRs are not the only receptors involved in this response however, as the C-type lectin CD205 was also required for optimal IFN-I production by SEA-stimulated cells.

IFN-I proved critical to the ability of Flt3L-generated cDCs (FL-cDCs) to polarise responses following adoptive transfer, as IFN-I receptor-deficient (*Ifnar1*$^{-/-}$) cells failed to prime an SEA-specific Th2 response in the dLN. *Ifnar1*$^{-/-}$ cells were almost completely unresponsive to SEA stimulation, failing to upregulate co-stimulatory molecules on their surface or to produce IFN-I. However *Ifnar1*$^{-/-}$ FL-cDCs displayed no deficiency in their ability to initiate T cell proliferation or IL-4-dependent Th2 polarisation *in vitro*. As T cell priming was abrogated *in vivo* only, this suggests that optimal cDC migration may be abrogated in the absence of the IFN-I receptor, although this is yet to be demonstrated definitively.

The importance of IFN-I responsiveness for optimal Th2 induction during helminth infection was also assessed. Th2 responses were normal in the liver of *S. mansoni Ifnar1*$^{-/-}$ mice; however, IL-4 and IL-13 levels in the mesenteric LN (MLN) were drastically reduced. It was found that Th2 induction in the MLN was also ablated in mice infected with the gastrointestinal helminth *Heligomosoides polygyrus*. This suggests that there is a selective dependency on IFN-I for the activation of Th2 responses in lymphoid organs.

The small intestine and the MLN provided an ideal site for further investigation of the development of the schistosome-specific immune response in peipheral tissues versus the draining lymph nodes, as this site is directly affected by parasite egg traffic during *S. mansoni* infection. The intestine is a unique immune environment – with a propensity towards regulation and tolerance, and a large population of innate effectors. Intestinal DCs depend on Flt3-L for their generation; however, the importance of DCs resident in the intestinal lamina propria (LP) for the initiation of Th2 inflammation in response to helminths is not yet known. Characterisation of LP DCs indicated that the activation of these cells is modulated during acute *S. mansoni* infection, whilst a novel model of schistosome egg deposition in the intestinal tissue illustrated that CD11c^{+} cells are essential for induction of the egg-specific Th2 response in both the LP and MLN following egg challenge.

These data demonstrate the importance of IFN-I signalling for the development of helminth-specific immune responses, highlighting for the first time a role for this pluripotent innate effector in Th2 induction. Development of an egg challenge model in the intestine also provides an ideal setting with which to further explore the importance of IFN-I for Th2 polarisation in peripheral tissues and lymphoid organs.

ABBREVIATIONS

AAMΦ: Alternatively-activated macrophage

Ag: Antigen

APC: Antigen presenting cell

ATP: Adenosine triphosphate

BDCA: Blood dendritic cell antigen

Batf3: Basic Leucine Zipper transcription factor, ATF-like 3

BM: Bone marrow

BMDC: Bone marrow derived dendritic cell

BMM: Bone marrow derived macrophage

CARD: Caspase recruitment domain

CCL: CC Chemokine Ligand

CCR: CC Chemokine receptor

CD: Complementarity Determining

cDC: Conventional DC

CDP: Common DC precursor

CLR: C-type lectin receptor

CpG: CpG oligodeoxynucleotide

CTLA-4: Cytotoxic T-lymphocyte antigen 4

CXCR: CXC chemokine receptor

DAMP: Danger associated molecular pattern

DC: Dendritic cell

Dectin1: Dendritic cell C-type lectin 1

dLN: draining lymph node

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

dsRNA: double-stranded ribonucleic acid

DSS: Dextran sulphate sodium

DTR: Diptheria toxin receptor

DTx: Diptheria toxin

ELISA: Enzyme linked immunosorbent assay

EOS: Eosinophil

ERK: Extracellular signal related kinase

FACS: Fluorescence activated cell sorting

FCS: Foetal-calf serum

FLDC: Flt3-L differentiated BMDC

Flt3: Fms like tyrosine kinase 3

Flt3-L: Fms like tyrosine kinase 3 ligand

Foxp3: Forkhead box protein 3

g: gram

GAS: Interferon-γ activated site

GBS: Group B streptococci

GFP: Green-fluorescent protein

GM-CSF: Granulocyte macrophage-colony stimulating factor

GMDC: GMCSF differentiated BMDC

GMFI: Geometric mean fluorescence intensity

GMP: Granulocyte macrophage progenitor

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HEV: High endothelial venule

HLA: Human leukocyte antigen

HPC: haematopoietic progenitor cell

HSC: haematopoietic stem cell

ICOS: Inducible costimulator

Id2: Inhibitor of DNA binding 2

IEC: Intestinal epithelial cell

IFN: Interferon

IFNAR: Interferon-alpha/beta receptor

IFN-I: Type I interferon

IFIT: IFN-induced protein with tetratricopeptide repeats

IGSF: Immunoglobulin super family

IKK: IκB kinase

IL: Interleukin

ILC: Innate lymphoid cell

ILF: Isolated lymphoid follicle

iNOS: Inducible nitric oxide synthase

i.p.: Intraperitoneal

IPSEα-1: IL-4-inducing principle of *S. mansoni* eggs alpha-1

IPS-1: Interferon-β promoter stimulator 1

IRAK: IL-1 receptor associated kinase

IRF: Interferon regulatory factor

ISG: Interferon-stimulated gene

ISGF3: Interferon-stimulated gene factor 3
ISRE: Interferon-stimulated response element
ITAM: Immunoreceptor tyrosine based activation motif
iTreg: peripherally induced FoxP3 expressing Treg
i.v.: Intravenous
JAK: Janus associated kinase
JNK: c-Jun N-terminal kinase
LGP2: Laboratory of genetics and physiology 2
LN: Lymph Node
LO: Lymphoid organ
LP: Lamina propria
LPS: Lipopolysaccharide
LCMV: Lymphocytic choriomeningitis virus
LNFPIII: Lacto-N-fucopentaose III
M-CSF: Macrophage- colony stimulating factor
MAL: MyD88-adaptor like
MAPK: Mitogen activated protein kinase
MDA5: Melanoma differentiation-associated gene 5
MDP: macrophage-DC precursor
MHC: Major Histocompatibility Complex
MKK: MAPK kinase
MKKK: MAPK kinase kinase
MKPs: MAPK phosphatases
MLN: Mesenteric lymph node
MLNX: Mesenteric lymphadenectomy
Mo-DC: monocyte-derived dendritic cell
MR: Mannose Receptor
mRNA: Messenger ribonucleic acid
miRNA: Micro-ribonucleic acid
Mx: Myxoma resistance protein
MyD88: Myeloid differentiation factor 88
MΦ: Macrophage
NFκB: Nuclear factor κ B
NK: Natural killer
NOD: Nucleotide oligomerisation domain
nTreg: natural Treg

°C: Degrees centigrade

Pa: Heat-killed *Propionibacterium acnes*

PAMP: Pathogen associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PD-L: Programmed death Ligand

pDC: Plasmacytoid dendritic cell

PDCA-1: Plasmacytoid denritic cell antigen-1

PD: Programmed death

pDC: Plasmacytoid DC

PGE_2: Prostaglandin E2

PI3K: phosphoinositide 3 kinase

pLN: Popliteal LN

PMA: Phorbol 12-myristate 13-acetate

PP: Peyers patch

PRR: pattern recognition receptor

R848: Resiquimod

RA: Retinoic acid

RAG: Recombination activating gene

RALDH: Retinal dehydrogenase

RANK: Receptor activator of NFκB

RIG: Retinoic acid inducible gene

RNase: Ribonuclease

RLR: RIG-like receptor

RNA: ribonucleic acid

RORγt: RAR-related orphan receptor gamma-t

RT-PCR: Real-time polymerase chain reaction

S1P: Sphingosine-1 phosphate

s.c.: Subcutaneous

SCF: Stem cell factor

SEA: Soluble egg antigen of *Schistosoma mansoni*

SI: Small intestine

SLO: Secondary lymphoid organ

St: *Salmonella typhimurium*

STAT: Signal transducer and activator of transcription

STING: Stimulator of interferon genes

Syk: Spleen tyrosine kinase

TAK: TGFβ activated kinase

TAP: Transporter associated with antigen processing

TBK-1: TANK binding kinase-1

TCR: T-cell receptor

TF: Transcription factor

Tfh: T follicular helper cell

Tg: Transgenic

TGFβ: Transforming growth factor beta

Th: T helper cell

Tip: TNFα and iNOS producing

TIR: Toll IL-1R homology domain

TIRAP: Toll-IL-1receptor domain containing adaptor

TLR: Toll like receptor

TNFα: Tumour necrosis factor alpha

TNFRSF: TNF receptor super family

Tr1: Foxp3 independent IL-10 dependent Treg

TRAF: TNFR associated factor 6

TRAM: TRIF related adaptor molecule

Treg: T regulatory cell

TRIF: Toll-IL-1receptor domain containing adaptor IFNβ

TSLP: Thymic stromal lymphopoietin

TYK: Tyrosine kinase

WT: Wildtype

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1.0 INTRODUCTION

The mammalian immune system is extraordinarily complex, involving numerous different types of cells capable of 1) recognising pathogen attack and mobilising a response, and 2) bringing the pathogen under control and ultimately guarding against future invasions. The cells responsible for orchestrating the first phase of this response comprise the innate immune system; cells of the adaptive immune system fulfil the second. The coordination required for such a system to work is gargantuan in scope and attests to a high degree of evolutionary sophistication.

1.1 Dendritic cells

Dendritic cells (DCs) are the sentinels of the immune system, providing an essential bridge between innate and adaptive immunity due to their ability to orchestrate T cell responses (Kapsenberg, 2003; Merad *et al.*, 2013). DCs reside in lymphoid tissues, but also in peripheral tissues at sites of potential pathogen entry, such as the skin and mucosal tissues (airways, lung, gastrointestinal and urogenital tracts) (Banchereau and Steinman, 1998). They are highly specialised antigen-presenting cells (APCs), capable of sensing, taking up and processing antigen (Ag), and presenting it to naïve T cells. Unlike other APC populations, such as macrophages (MΦs), DCs residing in peripheral tissues display enhanced migratory capacity following encounter with Ag and will migrate to the draining lymph node (dLN), where they can interact with naïve T cells (Banchereau and Steinman, 1998; Geissmann *et al.*, 2010a). The signals provided by DCs dictate the nature of the adaptive response by polarising T cells to a particular fate. Immunologists have known for some time the essential contribution of DCs to the immune system, and human health and disease (Collin *et al.*, 2012; Steinman, 2012). The importance of this cell type was highlighted to the world at large in 2012 with the posthumous award of the Nobel Prize for medicine or physiology to Prof. Ralph Steinman, who discovered DCs in 1973.

DCs were initially identified in mouse spleen as a cell type distinct from MΦs due to their dendritic morphology (Steinman, 2012; Steinman and Cohn, 1973). In time, it was shown that DCs express high levels of major histocompatibility complex (MHC) molecules on their surface (Steinman *et al.*, 1979). Some years later, the integrin CD11c was identified as a marker expressed at high levels on DCs that could be used to enrich for this cell

population (Metlay *et al.*, 1990). However, neither of these molecules provides a discriminating surface marker with which to identify DCs, as they are expressed on other cell types, such as B cells (MHC II), monocytes and MΦs (CD11c and MHC II). In particular, the overlapping expression profile of surface markers on DCs with MΦs and monocytes has been a complicating factor in the study of myeloid populations. The recent discovery of transcription factors (TFs) that are required during the differentiation of the DC lineage, and for development of specific subtypes, has begun to provide some clarity to those that study DC biology (Satpathy *et al.*, 2012b).

Broadly, DCs belong to two major subsets, conventional (or classical) DCs (cDCs) and plasmacytoid DCs (pDCs) (Merad and Manz, 2009). cDCs have two main functions: the maintenance of tolerance in peripheral tissues in the steady state, and the induction of specific immune responses against invading pathogens. The primary role of pDCs is the production of substantial amounts of type I interferon (IFN-I) in response to viral challenge or tissue damage (Colonna *et al.*, 2004; McKenna *et al.*, 2005).

1.1.1 Conventional DCs

For some years, researchers studying the role of DCs have made use of a depletable mouse model that depletes $CD11c^+$ cells; in this model all CD11c-expressing cells also express the transgene for the diptheria toxin receptor (CD11c-DTR) (Hochweller *et al.*, 2008; Jung *et al.*, 2002). There are two slightly different models: the original CD11c-DTR mice use a simian DTR transgene (Jung *et al.*, 2002), whilst CD11c-DOG mice use a human DTR inserted into a BAC construct (Hochweller *et al.*, 2008). Administration of diptheria toxin (DTx) leads to the selective loss of all $CD11c^+$ cells. The drawback of this model is that it is not selective for DCs; other CD11c-expressing cell populations are also ablated such as some MΦs, including marginal zone MΦs in the spleen, and monocytes (Hochweller *et al.*, 2008; Jung *et al.*, 2002; Probst *et al.*, 2005b; van Blijswijk *et al.*, 2013). There is variability in the depletion of DC subsets in the two models; in CD11c-DOG animals cDCs and pDCs are depleted, however, in CD11c-DTR mice pDCs are not affected, at least not in all tissues (Sapoznikov *et al.*, 2007). DTx treatment of CD11c-DTR mice also causes neutrophilia and monocytosis (Tittel *et al.*, 2012; van Blijswijk *et al.*, 2013). The recent discovery of the cDC-specific TF, Zbtb46, has provided a very useful tool to differentiate cells belonging to the cDC lineage from other myeloid cell populations

(Meredith *et al.*, 2012; Satpathy *et al.*, 2012a). Zbtb46 is also expressed by endothelial and erythroid cells, but in the immune system its expression is restricted to cDCs (Satpathy *et al.*, 2012a). Zbtb46 is expressed by committed cDC progenitors, all lymphoid tissue resident cDC subsets and cDCs in peripheral tissues (Meredith *et al.*, 2012; Satpathy *et al.*, 2012a). The TF is not expressed by pDCs, MΦs or monocyte-derived cells. By inserting the DTR transgene into the Zbtb46 promoter and creating bone marrow chimeras, a cDC-specific depletable mouse model has been developed (Meredith *et al.*, 2012; Satpathy *et al.*, 2012a), providing a more selective tool with which to study the role of cDCs.

Lymphoid tissue resident cDCs differentiate *in situ* from blood-borne precursors (Liu *et al.*, 2009; Liu *et al.*, 2007; Naik *et al.*, 2006) (Fig. 1.1), and remain in the lymphoid tissue for their entire lifespan (Merad *et al.*, 2013). Lymph node cDC populations also include nonlymphoid tissue migratory cDCs that enter via the lymphatics (Jakubzick *et al.*, 2008), whilst the spleen cDC population is comprised entirely of lymphoid resident cells (Randolph *et al.*, 2008). Lymphoid tissue cDCs primarily consist of two subsets: $CD8\alpha^+$ and $CD11b^+$ cDCs (Satpathy *et al.*, 2012a; Shortman and Heath, 2010)(Fig. 1.2). Other markers associated with the $CD8\alpha^+$ lineage include: CD24, and the C-type lectins Clec9a (DNGR1) and CD205 (Askew and Harding, 2008; Caminschi *et al.*, 2008; Jiang *et al.*, 1995; Sancho *et al.*, 2008). The TFs involved in the differentiation and maturation of $CD8\alpha^+$ cDCs are IFN Regulatory Factor (IRF)8, Id2 and Batf3 (Aliberti *et al.*, 2003; Hacker *et al.*, 2003; Hildner *et al.*, 2008; Tailor *et al.*, 2008). $CD8\alpha^+$ cDCs are specialised in their ability to cross-present Ag to $CD8^+$ T cells (den Haan *et al.*, 2000; Dudziak *et al.*, 2007; Hildner *et al.*, 2008; Schulz and Reis e Sousa, 2002).

$CD11b^+$ cDCs in lymphoid tissues can also be identified by high-level expression of Sirpα (Lahoud *et al.*, 2006). The development of $CD11b^+$ cDCs requires the TFs IRF2 and IRF4 (Ichikawa *et al.*, 2004; Suzuki *et al.*, 2004). $CD11b^+$ splenic cDCs also depend on Notch signalling for their development (Caton *et al.*, 2007; Lewis *et al.*, 2011). $CD8\alpha^+$ cDCs express the cellular machinery to facilitate Ag processing for cross-presentation to $CD8^+$ T cells (Dudziak *et al.*, 2007), including enriched levels of transporter associated with antigen processing (TAP) proteins, which are required for the loading of peptides onto MHC I (Joffre *et al.*, 2012). Studies in Batf3-deficient mice have demonstrated that $CD8\alpha^+$ cDCs

are uniquely required for cross presentation and activation of $CD8^+$ T cells during viral infection (Hildner *et al.*, 2008; Pinto *et al.*, 2011). In contrast, $CD11b^+$ cDCs are enriched in their expression of the components required for Ag processing and presentation to $CD4^+$ T cells, including cathepsins – proteases that breakdown Ag into peptide ligands for loading on MHC II (Dudziak *et al.*, 2007). $CD11b^+$ cDCs display an enhanced capacity to prime Ag-specific $CD4^+$ T cells when compared with $CD8\alpha^+$ cDCs (Dudziak *et al.*, 2007). In agreement with this, $CD11b^+$ cDCs preferentially present Ag to $CD4^+$ T cells (del Rio *et al.*, 2007), even during viral infection (Kim and Braciale, 2009). However, $CD8\alpha^+$ cDCs are also very good at activating $CD4^+$ T cells, by virtue of the fact that they express high levels of MHC II and produce large quantities of IL-12 (Shortman and Heath, 2010).

Tissue-resident cDCs account for around 1-5% of all cells in peripheral tissues, where the primary subsets are $CD103^+$ $CD11b^-$ and $CD103^-$ $CD11b^+$ populations (Merad *et al.*, 2013). There are also other tissue-specific, subsets that are present in the skin (Henri *et al.*, 2010) and the gastrointestinal tract (see Section 1.7). Tissue-resident $CD11b^+$ cDCs closely resemble their counterparts resident in lymphoid tissues, whilst $CD103^+$ cDCs are developmentally and functionally equivalent to $CD8\alpha^+$ cDCs (Bedoui *et al.*, 2009; Edelson *et al.*, 2010; Ginhoux *et al.*, 2009). $CD103^+$ cDCs, except in the intestine and pancreas, express the integrin langerin (Ginhoux *et al.*, 2009; Merad *et al.*, 2008). A subset of $CD103^+$ cDCs is also resident in the mesenteric lymph nodes (MLNs) (Johansson-Lindbom *et al.*, 2005; Satpathy *et al.*, 2013), and should not be confused with the migrating DC populations from the intestinal tissue.

Perhaps one criticism of the work concerning the identification of the different DC subsets and their ontogeny is that there is too much focus on characterisation and profiling with little functional readout. However, It could be argued that a clear understanding of the different subsets is required before their function can be assessed under different conditions. A comprehensive knowledge of DC ontogeny may also inform understanding of their function. For example, it has been shown that $CD103^+$ cDCs in peripheral tissues are developmentally related to splenic $CD8\alpha^+$ cDCs, suggesting that they may also be specialised in the cross-presentation of Ag, as had previously been reported (Bedoui *et al.*, 2009; Edelson *et al.*, 2010). Identification of the markers and TFs that are associated with the different DC subsets also enables the development of mouse models with targeted

deletion or depletion of specific subsets, tools that can be very useful for investigating function. This work also ensures that researchers are able to differentiate DCs from MΦs, and can make appropriate conclusions about the functions of these distinct cell populations.

1.1.2 Plasmacytoid DCs

pDCs are a small subset of DCs that share a similar origin to cDCs but have distinct lifecycle and functions. Unlike cDCs, which do not fully differentiate until they reach the tissues, pDCs develop in the bone marrow (BM) (Reizis, 2010). Both cDCs and pDCs develop from a common DC progenitor (CDP) (Naik *et al.*, 2007; Onai *et al.*, 2007), but cDC development requires a further intermediate: the pre-cDC (Diao *et al.*, 2006; Naik *et al.*, 2006). pDCs depend on expression of the TF E2-2, both for their differentiation in the BM, and for maintenance in the periphery (Cisse *et al.*, 2008; Ghosh *et al.*, 2010). Other TFs involved in pDC development include IRF8 and SpiB (Reizis *et al.*, 2011). In the steady state, pDCs are found primarily in lymphoid organs, entering from the blood (Cella *et al.*, 1999; Diacovo *et al.*, 2005). However, they are also found in surprisingly high numbers in the liver (Merad and Manz, 2009) and are rapidly recruited to sites of infection (Baldwin *et al.*, 2004; GeurtsvanKessel *et al.*, 2008; Smit *et al.*, 2006). Murine pDCs express only low-level MHC II and CD11c in the steady state, while other markers associated with this subset include CD45R (B220), PDCA-1, Ly6C, and the C-type lectins Siglec-H and Ly49Q (Reizis *et al.*, 2011).

The primary role of pDCs is thought to be as a source of IFN-I, particularly following viral infection, when they are involved in the early control of viral load and contribute to natural killer (NK) cell and $CD8^+$ T cell recruitment and activation (Pinto *et al.*, 2011; Swiecki *et al.*, 2010). However, despite their reputation for being key mediators of anti-viral immunity, in many viral infections including lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus, respiratory syncytial virus and herpes simplex virus, pDCs are not essential for the development of the immune response or for host survival (Reizis *et al.*, 2011). Mouse hepatitis is the only documented viral infection where the development of the immune response is pDC-dependent, as pDC IFN-I production is essential for control of viral expansion and host survival (Cervantes-Barragan *et al.*, 2007).

The ability of pDCs to act as APCs and prime naïve T cells is a contentious issue. A number of studies have suggested that pDCs can prime naïve T cells under certain circumstances, particularly when peptide Ag, which does not require further processing for presentation to T cells, is used (Irla *et al.*, 2010; Sapoznikov *et al.*, 2007). However, pDCs lack the cellular adaptations required to take up, process and present exogenous Ag effectively (Villadangos and Young, 2008). cDCs can regulate the cellular machinery involved in exogenous Ag presentation following activation: these modifications allow prolonged expression of MHC II on their surface, which is essential for optimal priming of $CD4^+$ T cells (Section 1.3)(LeibundGut-Landmann *et al.*, 2004; Young *et al.*, 2008). In contrast, pDCs do not have these adaptations and so are poor activators of naïve $CD4^+$ T cells (LeibundGut-Landmann *et al.*, 2004; Young *et al.*, 2008). However, unlike cDCs, pDCs retain the ability to present endogenous Ag following activation (Young *et al.*, 2008). Although pDCs accumulate in infected tissues, few migrate to the dLNs, and even then only relatively late after the onset of infection (GeurtsvanKessel *et al.*, 2008). Furthermore, since pDCs migrate to the dLNs via the blood rather than the afferent lymph (Cella *et al.*, 1999; Cerovic *et al.*, 2012; Diacovo *et al.*, 2005), it is unlikely that pDCs are directly involved in Ag transport to the dLNs. Instead, it is more likely that pDCs present Ag in the tissues to effector or memory T cells to enhance the efficacy of the immune response (GeurtsvanKessel *et al.*, 2008).

1.1.3 Monocyte-derived DCs

Monocytes derive from BM precursors, via a distinct pathway to DCs, and circulate in the blood in the steady state (Dominguez and Ardavin, 2010) (Fig. 1.1). They belong to two distinct subsets: patrolling monocytes ($Ly6C^{lo}$ $CX3CR1^{hi}$ $CCR2^{lo}$) and inflammatory monocytes ($Ly6C^{hi}$ $CX3CR1^{lo}$ $CCR2^{hi}$) (Geissmann *et al.*, 2008; Satpathy *et al.*, 2012b). Patrolling monocytes have a close association with the blood endothelium and so are rapidly recruited to sites of inflammation, where they provide a source of inflammatory mediators or aid in tissue repair (Auffray *et al.*, 2007; Geissmann *et al.*, 2008). Inflammatory monocytes are recruited to sites of inflammation with delayed kinetics compared to patrolling cells and are capable of differentiating into tumour necrosis factor- and inducible nitric oxide synthase- producing DCs (Tip-DCs), during *Listeria monocytogenes* infection and in response to some viral infections (Aldridge *et al.*, 2009; Geissmann *et al.*, 2008; Serbina *et al.*, 2003). These cells express low levels of CD11c

and have a DC-like morphology (Geissmann *et al.*, 2008). However, they do not express the cDC TF Zbtb46 (Meredith *et al.*, 2012; Satpathy *et al.*, 2012a), suggesting that these cells may simply be inflammatory MΦs or monocytes. Moreover, whilst both DCs and macrophages develop from a common precursor in the BM (MΦ-DC progenitors, MDPs) monocytes differentiate in response to specific cues via a distinct pathway (Geissmann *et al.*, 2010b). Thus, these cells are not DCs per se but may be capable of Ag presentation in specific tissues and under certain conditions (Kamphorst *et al.*, 2010), for example in the lung during viral infection (Aldridge *et al.*, 2009) and allergic Th2 responses (Hammad *et al.*, 2010; Plantinga *et al.*, 2013).

1.1.4 Flt3-L and GM-CSF: DC development versus homeostasis

Fms-like tyrosine kinase 3 ligand (Flt3-L) is the major cytokine responsible for DC generation and turnover *in vivo*. Whilst granulocyte-MΦ colony-stimulating factor (GM-CSF) can be used to differentate DCs *in vitro* (see Section 1.1.5), it does not seem to be essential for DC development *in vivo*. In the absence of GM-CSF or its receptor, there is only a minor reduction in cDC numbers and no change in the pDC population (Vremec *et al.*, 1997). Furthermore, the slight reduction in total DC numbers in the absence of GM-CSF appears to correlate with a concurrent reduction in spleen size and so probably does not reflect a specific defect in the DC compartment (Naik, 2008). In contrast, genetic deletion of Flt3-L leads to a dramatic loss of DC populations in all lymphoid and peripheral tissues, with approximately 90% reduction in DC numbers in the spleen and LN of Flt3-L deficient animals (Kingston *et al.*, 2009; McKenna *et al.*, 2000). pDCs display the most striking reduction in the absence of Flt3-L (Kingston *et al.*, 2009; McKenna *et al.*, 2000). Both MDPs and CDPs in the BM express Flt3 (D'Amico and Wu, 2003; Fogg *et al.*, 2006; Naik *et al.*, 2007; Onai *et al.*, 2007), and both populations are depleted in the absence of Flt3-L (Kingston *et al.*, 2009). Pre-DCs in the periphery are also $Flt3^+$ and depend on Flt3-L for their further differentiation into the cDC subsets (Liu *et al.*, 2009; Waskow *et al.*, 2008). Flt3 signals via signal transducer and activator of transcription (STAT) 3, thus STAT3 expression is also essential for DC development (Laouar *et al.*, 2003).

The combined loss of Flt3-L and GM-CSF leads to a more dramatic loss of cDC numbers in peripheral tissues compared to the reduction seen in mice that only lack Flt3-L (Kingston *et al.*, 2009). In the absence of Flt3-L signalling, there is a 35% reduction in dermal DC

numbers, whereas in the skin of double-deficient animals, cDCs are reduced by approximately 75%. These findings are in agreement with recent suggestions that GM-CSF is involved in cDC homeostasis in the periphery (Greter *et al.*, 2012; King *et al.*, 2010). In the absence of GM-CSF, there is a reduction of $CD103^+$ and $CD11b^+$ cDCs numbers in the lung, skin and intestine (Greter *et al.*, 2012; King *et al.*, 2010; Kingston *et al.*, 2009). GM-CSF is required for the survival of cDCs in the periphery: in the absence of GM-CSF signalling, cDCs are more pro-apoptotic, showing increased expression of caspase-3 and signs of mitochondrial fragmentation, as well as an increase in cells actively undergoing apoptosis (Greter *et al.*, 2012). Signalling downstream of GM-CSF activates STAT5 followed by signalling pathways involved in DC maturation, activation and survival, including the NFκB and MAPK pathways (van de Laar *et al.*, 2012). It is likely that these signalling events are required to maintain healthy cDCs in the periphery. In contrast, ligation of Flt3 activates STAT3 only (Laouar *et al.*, 2003), with no impact on the NFκB pathway, therefore Flt3-L does not stimulate DC activation in the same way as GM-CSF (van de Laar *et al.*, 2012). GM-CSF is also required for the expression of CD103 by tissue-resident cDCs (Edelson *et al.*, 2011; Greter *et al.*, 2012; Zhan *et al.*, 2011) and it has been suggested that CD103 expression may be required for full differentiation of this subset and for their capacity to cross-present Ag (Sathe and Wu, 2011; Zhan *et al.*, 2011). Unlike cDCs, pDCs do not depend on GM-CSF, in fact, activation of STAT5 by GM-CSF actively inhibits pDC differentiation (Esashi *et al.*, 2008).

1.1.5 DCs generated *in vitro*

Due to the scarcity of DCs *in vivo* (~1% of all cells in lymphoid tissues in the steady state (Merad and Manz, 2009)), many studies of DC biology make use of DCs generated *in vitro* from BM. The most commonly used BMDCs are those generated with GM-CSF (GMDCs) (Inaba *et al.*, 1992; Lutz *et al.*, 1999). Large numbers of DCs can be generated *in vitro* using GM-CSF, as differentiating cells proliferate in culture (Lutz *et al.*, 1999). GMDCs are homogenously $CD8\alpha^-$ and express high levels of CD11b (Brasel *et al.*, 2000), however GMDCs are more closely related to monocytes and MΦs than $CD11b^+$ cDCs in terms of their mRNA expression profile (Robbins *et al.*, 2008). It has been suggested that GMDCs are more analogous to TipDCs or monocyte-derived DCs that develop under inflammatory conditions (Xu *et al.*, 2007). However, TipDCs do not express the cDC-specific TF Zbtb46 – most likely because they are monocytes/ MΦs rather than DCs, whereas GMDCs do

(Meredith *et al.*, 2012; Satpathy *et al.*, 2012a). Moreover, TipDCs develop in the absence of GM-CSF (Greter *et al.*, 2012), demonstrating that these cells are entirely distinct from GMDCs generated *in vitro*. Given that GM-CSF is not involved in DC differentiation *in vivo*, it is perhaps not surprising that there is not a direct *in vivo* counterpart for GMDCs. Nonetheless, GMDCs have afforded researchers an invaluable tool with which to study DC biology and function.

The fact that GM-CSF gives rise to a homogenous population of DCs is advantageous as it means that cells do not have to be sorted for full characterization of their phenotype and function. GM-CSF generates large numbers of cells that are generally in a low-activation state (i.e. expressing low-levels of MHC II, if grown carefully). The main advantage of these cells as a model for studying DCs, is that they most closely resemble cells that are generated from human blood-derived monocytes, using GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994), enabling direct comparisons between these cell types.

Generation of BMDCs using Flt3-L gives rise to mixed cultures of pDCs and the cDC subsets, which are thought to be equivalent to those found in lymphoid organs *in vivo* (Naik *et al.*, 2005; Naik *et al.*, 2007). Although Flt3-L generated cDCs (FL-cDCs) do not express CD8α, an equivalent population can be identified that is $CD24^{hi}$ and $CD11b^{lo}$ (Naik *et al.*, 2005). Similarly to their *in vivo* counterparts, $CD11b^{+}$ FL-cDCs express high levels of Sirpα (Naik *et al.*, 2005). pDCs generated *in vitro* using Flt3-L have a similar expression profile of surface markers to their *in vivo* counterparts, with high levels of CD45R and Ly6C, and little or no CD11b on their surface (Brawand *et al.*, 2002; Naik *et al.*, 2005). FL-pDCs are also capable of secreting high levels of IFN-I in response to Ag stimulation (Brawand *et al.*, 2002; Naik *et al.*, 2005), whilst $CD24^{+}$ FL-cDCs alone display the ability to cross-present to $CD8^{+}$ T cells (Naik *et al.*, 2005). Similarly to their splenic counterparts, $CD24^{+}$ cDCs cannot be generated from IRF8-deficient BM (Naik *et al.*, 2005), whilst IRF4-deficient cultures display a reciprocal deficit in $CD11b^{+}$ cDCs (Suzuki *et al.*, 2004). Both FL-cDC subsets express Zbtb46 (Meredith *et al.*, 2012; Satpathy *et al.*, 2012a). Thus, FLDCs provide a unique tool with which to study equivalents of all the major DC subsets present *in vivo* in the steady state.

1.1.6 DC migration from peripheral tissues to the dLN

In order to interact with and prime naïve T cells, DCs that encounter Ag whilst patrolling in the peripheral tissues must first migrate to the dLN. cDCs leave the peripheral tissues and migrate via the afferent lymphatics (Alvarez *et al.*, 2008). Much of our understanding of cDC migration comes from studies of DCs trafficking from the skin to the dLN, which demonstrate that successful transit is dependent on the chemokines CCL19 and CCL21, and their receptor CCR7 (MartIn-Fontecha *et al.*, 2003; Saeki *et al.*, 1999). Exposure to Ag and inflammatory mediators, such as IL-1β and TNFα, provides a strong stimulus for DCs to leave the tissues. However, a small number of DCs migrate constitutively in the steady state (Alvarez *et al.*, 2008; Jakubzick *et al.*, 2008). Although immature cDCs express only low-levels of CCR7 (Ricart *et al.*, 2011; Ritter *et al.*, 2004), the receptor and its ligands are still thought to be the primary mediators of steady state migration, as *Ccr7*$^{-/-}$ mice have a 30-40% reduction in their LN DC populations in the absence of any inflammatory stimulus (Ohl *et al.*, 2004).

CCR7 expression is required for transmigration into the lymphatic lumen, enabling cDCs to dock with CCL21 on the lymphatic endothelium and enter via pores within the discontinuous laminin-expressing basement membrane, before crawling and paracellular entry, at junctions of lymphatic endothelial cells into the capillary lumen, (Pflicke and Sixt, 2009; Tal *et al.*, 2011). Once inside the initial lymph vessels, cDCs actively crawl along the endothelium, and are then transported passively in collecting lymphatics where the flow rate is higher (Tal *et al.*, 2011). Although DC crawling requires engagement of adhesion molecules such as integrins, directional movement may still depend on CCR7-CCL21 interactions (Shields *et al.*, 2007). It is also thought that migrating DCs gain entry to the LN by following a gradient of CCL21, which is expressed at high levels in the LN paracortex (Nakano and Gunn, 2001). In support of this, in the absence of CCL21, DCs are unable to gain entry to the LN and instead accumulate in the subcapsular sinus on the outer edge of LNs (Mori *et al.*, 2001). Clearly, CCR7 has an integral role at several stages of cDC migration from peripheral tissues to the draining LN.

As highlighted above (section 1.1.2), pDCs do not migrate from the tissues to lymphoid organs in the lymph, but rather via the blood. An early study of pDCs indicated that these cells were highly concentrated around high-endothelial venules (HEV) in LNs (Cella *et al.*,

1999), suggesting that they may enter via this route. pDCs can adhere to non-inflamed HEV, but it is only under inflammatory conditions, when endothelial cells upregulate CD62L, that they can transmigrate (Diacovo *et al.*, 2005). Studies of CCR5-deficient cells indicate that this process requires pDC expression of CCR5 (Diacovo *et al.*, 2005).

1.1.7 Human DC subsets

Most of our understanding of human DC subsets stems from studies of the DC subsets generated from, or found in, the blood due to the scarcity of samples from tissues. Human pDCs are lineage-negative ($CD3^-$ $CD14^-$ $CD19^-$ $CD20^-$ $CD56^-$) and do not express CD11c, but are constiutively MHC II^+ (Reizis *et al.*, 2011). Other markers used to identify pDCs in humans include blood dendritic cell Ag (BDCA) 2, BDCA4 and CD123 (IL-3Rα) (Merad *et al.*, 2013; Reizis *et al.*, 2011). Human cDCs in the blood are identified as lineage-negative, but positive for CD11c and MHC II (Merad *et al.*, 2013). In contrast to mice, in which circulating pre-DCs differentiate into mature cDC subsets in the periphery, human blood contains two fully differentiated cDC subsets that can be distinguished by their expression of the non-overlapping surface markers, CD1c (BDCA1) and CD141 (BDCA3) (Lindstedt *et al.*, 2005; Merad *et al.*, 2013). $CD1c^+$ DCs are by far the predominant population in human blood (Mittag *et al.*, 2011; Nizzoli *et al.*, 2013)(Fig. 1.2).

$CD141^+$ human DCs are thought to be equivalent to the $CD8\alpha^+$/ $CD103^+$ subset in mice (Jongbloed *et al.*, 2010; Poulin *et al.*, 2010; Robbins *et al.*, 2008) (Fig. 1.2). As with $CD8\alpha^+$ cDCs in mouse, $CD141^+$ human DCs express Clec9a on their surface (Jongbloed *et al.*, 2010; Poulin *et al.*, 2010). Both murine $CD8\alpha^+$ cDCs and human $CD141^+$ DCs display an enhanced ability to internalise apoptotic cells, and are the primary producers of IL-12 in response to TLR ligands (Iyoda *et al.*, 2002; Jongbloed *et al.*, 2010; Poulin *et al.*, 2010; Reis e Sousa *et al.*, 1997). Like $CD8\alpha^+$ cDCs in mouse, human $CD141^+$ DCs efficiently cross-present to $CD8^+$ T cells (Jongbloed *et al.*, 2010; Poulin *et al.*, 2010). It should be noted, however, that the $CD1c^+$ subset can also cross-present and activate $CD8^+$ T cells efficiently (Mittag *et al.*, 2011; Nizzoli *et al.*, 2013). As described above, development of murine $CD8\alpha^+$/ $CD103^+$ cDCs depends on the TF Batf3 (Edelson *et al.*, 2010; Hildner *et al.*, 2008). Differentiation of human DCs *in vitro* from haematopoietic stem cells (HSCs)/ haematopoietic progenitor cells (HPCs) using a combination of Flt3-L and GM-CSF gives rise to a subset of $CD141^+$ $Clec9a^+$ DCs (Poulin *et al.*, 2010). Blockade of Batf3 in these

cultures abolishes the development of this subset (Poulin *et al.*, 2012), highlighting the developmental similarities of human $CD141^+$ DCs to murine $CD8\alpha^+$ cDCs.

Due to the restrictively small number of DCs that can be purified from human blood, DCs are often generated *in vitro* from $CD14^+$ monocytes that are present at much higher numbers in human blood (Merad *et al.*, 2013). GM-CSF is the primary differentiation factor used in these cultures, with the addition of different cytokines impacting on the phenotype of the DCs that are generated. GM-CSF together with IL-4 gives rise to a homogeneous population of monocyte-derived DCs (mo-DCs) that are $CD1a^+$ and relatively immature, in terms of their surface expression of MHC and co-stimulatory molecules (Palucka *et al.*, 1998; Sallusto and Lanzavecchia, 1994). Mo-DCs generated with GM-CSF/ IL-4 also efficiently present Ag to T cells (Sallusto and Lanzavecchia, 1994). An alternative method for generating DCs uses HSCs/ HPCs from cord blood, rather than monocytes from venous blood. This culture method is more complex and involves two steps. Initially, HSCs/ HPCs are cultured for 7-11 days with Flt3-L, in combination with stem cell factor (SCF), IL-3 and IL-6 (Poulin *et al.*, 2010). Cells are then re-cultured for the final 12-14 days of culture with Flt3-L, SCF, GM-CSF and IL-4 (Poulin *et al.*, 2010). The DCs generated from these cultures include pDCs, $CD1c^+$ and $CD141^+$ cDCs - a major advantage of this method (Merad *et al.*, 2013; Poulin *et al.*, 2010). These cells also most closely resemble the DCs generated from murine BM *in vitro* using Flt3-L.

1.2 DC Recognition of non-self and danger

DCs express a range of pattern recognition receptors (PRRs) that have evolved to detect specific pathogen-associated molecular patterns (PAMPs) (Janeway, 1989; Kapsenberg, 2003). Although it was originally presumed that PRRs would selectively recognise non-self, they are also responsive to endogenous ligands, often components of damaged or necrotic cells, such as nucleic acids or heat-shock proteins, which act as danger signals following tissue damage (damage-associated molecular patterns, DAMPs) (Bianchi, 2007; Matzinger, 2002). It has been suggested that, although self-ligands can bind to the same receptors as PAMPs, the outcome of receptor ligation by danger signals may be distinct, initiating wound repair and healing rather than immune activation (Iwasaki and Medzhitov, 2010). The PRRs expressed by DCs can broadly be categorised by their cellular localisation. The transmembrane PRRs include Toll-like receptors (TLRs) and C-type lectin

receptors (CLRs). A more recently identified class of PRRs are nucleic acid sensors found in the cytosol which include the retinoic acid-inducible gene I (RIG-I) -like receptors (RLRs).

1.2.1 TLRs

TLRs, which recognise an expansive range of PAMPs as well as DAMPs, were the first PRR family to be identified (Lemaitre *et al.*, 1996; Vogel, 1998). TLRs are localised either to the plasma membrane or on endosomal/lysosomal membranes, with the surface membrane receptors primarily recognising microbial patterns that are expressed on the cell surface, whilst the intracellular TLRs recognise nucleic acid ligands (Table 1.1). Many TLRs require co-receptors to function fully, for example in order to recognise LPS, TLR4 requires the myeloid differentiation factor 2 (MD2) as a co-receptor, which associates with the TLR4 homodimer on the cell surface or in the endolysosome (Shimazu *et al.*, 1999). A number of synthetic TLR ligands have been developed that are recognised by specific TLRs. Synthetic ligands facilitate characterisation of cellular responses to the stimulation of a specific TLR in isolation, without the complexity of microorganisms, which often incorporate many different antigenic patterns. Synthetic ligands can also be used, or have the potential to be used, as adjuvants to enhance vaccine efficacy. Polyinosinic:polycytidylic acid (pI:C) is structurally similar to dsRNA and selectively activates TLR3. Resiquimod (R848) belongs to a family of anti-viral compounds that are agonists for TLR7 and TLR8 (Hemmi *et al.*, 2002). Bacterial DNA that contains unmethylated CpG dinucleotides is highly immunostimulatory (Yamamoto *et al.*, 2000), and immune activation in response to purified native or synthetic CpG is dependent on TLR9 (Hemmi *et al.*, 2000).

1.2.2 DC expression of TLRs

DC subsets in human and mouse have distinct expression patterns of TLRs (Table 1.2 and 1.3), which not only confers selective sensitivity to specific pathogens, but is also associated with the function of different DC populations. For example, pDCs selectively express very high levels of TLR7 and 9, allowing them to respond preferentially to viral challenge (Diebold *et al.*, 2004; Takeuchi and Akira, 2010). Similarly, TLR3 is expressed only by $CD8\alpha^+$ cDCs in mice (Jelinek *et al.*, 2011) and in the $CD141^+$ DC population in humans (Hemont *et al.*, 2013; Jongbloed *et al.*, 2010; Poulin *et al.*, 2010), which seems to

relate to the ability of these cell subsets alone to cross-present to $CD8^+$ T cells. Although the expression patterns of human mo-DCs, pDCs and mDCs collectively have been known for a while (Iwasaki and Medzhitov, 2004; Schreibelt *et al.*, 2010), it is only very recently that the specific expression patterns of $CD1c^+$ and $CD141^+$ DCs have been elucidated (Hemont *et al.*, 2013) (Table 1.3).

1.2.3 TLR adaptor proteins and downstream signalling

Transcriptional activation occurs following ligation of TLRs, the specific genes that are activated being dependent on which TLRs are engaged and the adaptor proteins that are recruited. MyD88 is the adaptor protein for most TLRs, except for TLR3, which selectively recruits TRIF (Yamamoto *et al.*, 2003). TLR4 signal transduction can also occur in a MyD88-independent manner via TRIF leading to IFN-β production (Yamamoto *et al.*, 2002). It is thought that activation of these two signalling pathways by TLR4 can occur sequentially (Takeuchi and Akira, 2010). The signalling events that occur downstream of MyD88 and TRIF are illustrated in Fig. 1.3.

1.2.4 Activation of IFN-I expression downstream of TLRs

Activation of both MyD88- and TRIF-dependent pathways in response to nucleic acid ligands triggers downstream signalling events leading to the stimulation of IFN-I gene expression (Honda and Taniguchi, 2006a). Transcription of IFN-I genes following TLR ligation is dependent on two IRFs – IRF3 and IRF7 - without which recruitment of chromatin modifiers and the transcriptional machinery to the promoter do not occur (Honda and Taniguchi, 2006a; Honda *et al.*, 2005b). IRFs are activated by two kinases, TANK-binding kinase (TBK)-1 and IKKε (Tseng *et al.*, 2010)(Fig. 1.3). All IRFs contain a highly-conserved DNA-binding domain, which recognizes a consensus DNA sequence known as the IFN-stimulated response element (ISRE) (Honda and Taniguchi, 2006b). This domain is found in the promoters of genes that sequence the IFN-I subsets themselves, as well as many other genes involved in immunity. IRF3 is constitutively expressed and resides in the cytosol in a latent state. Unlike IRF3, IRF7 is expressed only at low levels in most cells but is strongly induced by IFN-I signalling. IRF3 and IRF7 are highly homologous, and can form homo- or hetero-dimers following phosphorylation prior to translocation into the nucleus to initiate gene expression of IFN-I and other IFN-stimulated genes (ISGs) (Honda and Taniguchi, 2006b; Takeuchi and Akira, 2010).

MyD88 activates IFN-I production via a distinct signalling pathway downstream of TLR9. MyD88 interacts directly with IRF7 in the endosomal compartment. Following ligation of TLR9, IRF7 also interacts with TRAF6 and is activated in a MyD88-TRAF6 dependent manner (Honda *et al.*, 2004; Kawai *et al.*, 2004). It is likely that IRAKs and TRAF3 are also involved in this process, though their roles are less clear (Honda and Taniguchi, 2006b). Robust IFN-I gene induction in splenic pDCs is normal in IRF1 and IRF3-deficient mice, however this response is ablated in IRF7-deficient animals, demonstrating that IRF7 is the primary mediator of IFN-I in pDCs (Honda *et al.*, 2005b). It was initially assumed that pDCs are able to produce such large amounts of IFN-I, unlike cDCs and other cells, because they constitutively express IRF7 to a higher level (Izaguirre *et al.*, 2003; Prakash *et al.*, 2005). However, it is clear that spatio-temporal control of TLR9 signalling in pDCs also plays a role. In pDCs, CpG ligands are maintained in the endosome with the MyD88-IRF7 complex for long periods, whilst in cDCs, CpG is rapidly transferred to lysosomal vesicles (Honda *et al.*, 2005a).

1.2.5 Negative regulation of TRIF function by alternative adaptors

Activation of the adaptor proteins and their downstream signalling pathways is tightly controlled to prevent prolonged or unnecessary expression of pro-inflammatory mediators (Takeuchi and Akira, 2010). There is increasing understanding of these processes, which include negative regulation of specific adaptor proteins by other adaptors. Many of these interactions involve the downmodulation of TRIF signalling pathways. The most recently identified adaptor protein containing a TIR domain is SARM, which interacts directly with TRIF to inhibit NFκB and IRF-dependent gene expression (Carty *et al.*, 2006). Since this interaction was identified, it has also been shown that MyD88 can negatively regulate TRIF, via two distinct mechanisms.

The first of these was shown in a pI:C-dependent model of corneal inflammation, where the inflammatory response is activated downstream of TLR3-TRIF signalling, that MyD88-deficient animals had exacerbated inflammation, including an increase in MΦ infiltration and CCL5 production (Johnson *et al.*, 2008). MyD88 regulation of this pathway did not influence IRF3 function or directly act on NFκB. However, the MAP kinase c-Jun N-

terminal kinase (JNK) was involved, as MyD88 knockdown significantly increased JNK phosphorylation in human corneal cells.

MyD88 has also been shown to directly regulate IRF3 activity downstream of TRIF. In this pathway MyD88 associates with IRF3, preventing its phosphorylation by IKKε and resulting in reduced IFNβ production by pI:C-treated BMMΦs, however MyD88 did not affect TBK-1 activity (Siednienko *et al.*, 2011). MyD88 inhibition of TRIF-IRF3 activation was also shown to inhibit CCL5 production by BMMΦs following their exposure to pI:C. MyD88-adaptor like (MAL) can also inhibit TRIF-mediated IFNβ production, albeit by a distinct pathway that does not affect CCL5. MAL associates with IRF7, inhibiting its phosphorylation and nuclear translocation (Siednienko *et al.*, 2011). Thus, TRIF signalling pathways are controlled and downmodulated by a range of adaptor proteins using distinct mechanisms.

1.2.6 Cytosolic nucleic acid sensors: The classical pathway of IFN-I induction

It was shown some time ago that viral infection induces IFN-I production from a range of cell types, including non-immune cells, such as epithelial cells. This response is mediated by cytosolic PRRs that recognise nucleic acids (Desmet and Ishii, 2012). Cytosolic nucleic acid sensors are a large and growing family of PRRs, the best characterised of which are the RLRs and Nod-like receptors (NLRs). Of these, RLRs are more important for IFN-I production, particularly by myeloid cells, epithelial cells and cells of the central nervous system (Honda and Taniguchi, 2006a). Although RLRs are expressed by pDCs, they are not essential for IFN-I production by this cell type (Loo and Gale, 2011). The RLR family includes three members: RIG-I, MDA5 and LGP2. RIG-I and MDA5 consist of two N-terminal CARD domains, a DExD/H-box RNA helicase domain and a C-terminal repressor domain (Takeuchi and Akira, 2009). It is the C-terminal regulatory domain of RLRs that bind nucleic acid ligands. RLRs catalyse ATP via the DExD/H helicase domain. This ATPase function is essential for IFN-I production by RLRs in all settings (Takeuchi and Akira, 2010). LGP2 lacks the CARD domains, which are essential for activating downstream signalling pathways (Loo and Gale, 2011). LGP2 is thought to be a positive regulator of the other two family members (Satoh *et al.*, 2010). It has been shown that RIG-I can bind short ssRNA and dsRNA, with the presence of a 5’ triphosphate domain greatly increasing IFN-I production downstream of RIG-I, whilst MDA5 is activated by long

dsRNA (Desmet and Ishii, 2012; Takeuchi and Akira, 2010). AT-rich dsDNA can indirectly activate RIG-I, leading to IFN-I production, a process that is mediated by cytosolic RNA polymerase III, which utilises the DNA as a template for transcription to generate 5-ppp ssRNA that is detected by RIG-I (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). RIG-I and MDA5 can also recognise short self-RNAs that are cleaved by RNase L, and activation by these ligands can enhance IFN-I production during viral infection (Malathi *et al.*, 2007).

The adaptor protein downstream of RLRs is Interferon-β promoter stimulator 1 (IPS-1, also known as MAVS, CARDIF, VISA), which is located within the mitochondrial membrane (Kato *et al.*, 2011; Kawai *et al.*, 2005). Like RIG-I and MDA5, IPS-1 contains a CARD domain, with which it interacts with the cytosolic receptors (Kato *et al.*, 2011; Kawai *et al.*, 2005). Activation of IPS-1 is essential for IFN-I production downstream of RLRs following exposure to RNA ligands, via TBK-1 and IKKε activation of IRF3 and IRF7 (Desmet and Ishii, 2012). The signalling events downstream of RLR ligation (and other cytosolic nucleic acid sensors) are shown in Fig. 1.4.

RLRs are not the only cytoplasmic RNA helicases that recognise nucleic acid ligands. An increasing number of other DExD/H-box family members are being identified, which recognise a diverse range of nucleic acid ligands and utilise a variety of adaptor proteins. For example, the helicases DDX1, DDX21 and DHX36 form a complex that recognises dsRNA, and stimulates IFN-I induction in response to pI:C stimulation or viral infection (Zhang *et al.*, 2011a). This interaction was demonstrated in GMDCs and is dependent on the adaptor molecule TRIF. A number of other cytosolic helicases utilise the RLR-associated adaptor protein IPS-1 to activate IFN-I gene expression (Desmet and Ishii, 2012), such as DDX3, which is constitutively expressed and recognises viral RNA (Oshiumi *et al.*, 2010). Another adaptor molecule activated by these receptors is STING, which is associated with the endoplasmic reticulum and is also capable of IFN-I induction (Broz and Monack, 2013; Desmet and Ishii, 2012). One such cytosolic sensor that utilises this adaptor is the helicase DDX41, which was shown to recognise intracellular DNA in GMDCs, activating IFNβ production downstream of STING (Zhang *et al.*, 2011b) (Fig. 1.4).

1.2.7 CLRs

C-type lectin receptors can recognise a wide range of ligands, both pathogen-associated and self. They have been shown to play an important role in directly activating gene expression, as well as modulating the action of TLRs (Geijtenbeek and Gringhuis, 2009). CLRs are a group of receptors that are calcium-dependent and share a common carbohydrate-recognition domain, or C-type lectin-like domain (Kerrigan and Brown, 2010). A wide-range of CLRs are expressed by DCs and some are specifically associated with certain DC subsets. For example, Clec9a is expressed selectively by $CD8\alpha^+$ cDCs in mouse (Caminschi *et al.*, 2008), and $CD141^+$ DCs that are the human equivalent of this subset (Poulin *et al.*, 2010). The CLRs expressed by DCs that act as PRRs primarily recognise mannose, fucose and glucan carbohydrate structures, which allows the recognition of a range of pathogens, including bacteria, viruses, fungi, and helminth parasites (Geijtenbeek and Gringhuis, 2009). Ligand-binding to CLRs leads to receptor clustering and to receptor phosphorylation by Src-family kinases, providing a docking site for the adaptor protein Syk, which initiates downstream signalling mediated by CARD9 (Gross *et al.*, 2006), including activation of the NFκB pathway (Kerrigan and Brown, 2010). Although CLRs can function in isolation to activate signalling pathways, it has also been shown that the CLR Dectin-1 can collaborate with TLRs 2, 4, 5, 7 and 9 to induce production of TNFα, CCL3 and CCL4 in an NFκB-dependent manner (Dennehy *et al.*, 2008), providing further evidence that PRRs should not only be considered in isolation.

1.2.8 NFκB signalling in DCs

The mammalian nuclear factor-κB (NFκB) family consists of five TFs that regulate inducible gene expression in a range of physiological processes. They play an essential role in orchestrating the activation of immune cells during an immune response and are integral to DC responses to Ag stimulation via PRRs, as well as cytokine signals (Hayden *et al.*, 2006), such as GM-CSF. NFκB family members must dimerise to become functional, allowing the family to bind and modulate the transcription of a diverse range of genes (Gilmore, 2006).

A family of inhibitory IκB proteins, which sequester inactive dimers in the cytoplasm, tightly regulates NFκB activity (Ghosh and Hayden, 2008). When the pathway is activated, the IκB protein is degraded and the NFκB complex enters the nucleus to modulate gene

transcription. The IKK complex consists of two kinases, IKKα and IKKβ, and the regulatory scaffold protein, IKKγ (NEMO) (Lawrence, 2009; Scheidereit, 2006). In the canonical NFκB pathway, IKKβ and IKKγ are required for the activation of NFκB complexes, whilst the non-canonical pathway depends on the activity of IKKα (Hayden *et al.*, 2006; Lawrence, 2009). Signalling downstream of MyD88 and TRIF, and other adaptor proteins, leads to the activation of canonical IKKβ-dependent NFκB complexes (Hayden *et al.*, 2006). Binding of IL-1 and TNFα to their cognate receptors also activates the canonical NFκB pathway, while activators of the alternative pathway include CD40L binding to CD40 (Lawrence, 2009).

Little is known about the activation of signalling pathways in DCs following exposure to helminth Ag. It has been shown that NFκB1 is required for DC induction of Th2 responses (Artis *et al.*, 2005), whilst others have demonstrated activation of the MAPK pathway following exposure to helminth Ags (Kane *et al.*, 2004; Thomas *et al.*, 2003). Yet, there remains much still to be uncovered about the role of these pathways in the polarisation of DCs towards Th2 induction.

1.3 Antigen presentation by DCs

The primary function of DCs is to process and present Ag to naïve T cells and to direct polarisation of Ag-specific adaptive responses (Kapsenberg, 2003; Steinman, 2012; Walsh and Mills, 2013). Key to this is the expression of MHC molecules on the surface of DCs, glycoprotein complexes that can bind and present peptide Ag to T cells (Villadangos and Schnorrer, 2007). MHC I complexes can be found on the surface of any nucleated cell and present Ag to $CD8^+$ T cells (Neefjes *et al.*, 2011). Expression of MHC II is restricted primarily to DCs, MΦ and B cells in the periphery, although it is also expressed by thymic epithelial cells (Li *et al.*, 2005). During infection, MHC II can be upregulated on the surface of other innate cell populations, including basophils (Perrigoue *et al.*, 2009; Sokol *et al.*, 2009) and eosinophils (Dardalhon *et al.*, 2008; Shi, 2004). Ags on MHC II complexes are presented to $CD4^+$ T cells, leading to the induction of T helper (Th) and T regulatory (Treg) cells (Kapsenberg, 2003; Villadangos and Schnorrer, 2007).

With PRRs localised on the cell surface, in endosomes and within the cytosol, Ags both from within the cell (endogenous), and from the extracellular environment (exogenous) can

be sensed by DCs. It was originally thought that MHC I molecules presented only endogenous Ag, whilst MHC II bound and presented exogenous Ag (Neefjes *et al.*, 2011). However, it has been shown in recent years that both classes of MHC molecules can present all forms of Ag. Analysis of MHC II-bound peptides has shown that a substantial number is derived from the cytosol (Dongre *et al.*, 2001; Rudensky *et al.*, 1991), indicating that it is not only MHC I molecules that present this class of Ag. Exogenous Ags can be presented on MHC I via cross-presentation, a function that is uniquely performed by $CD8\alpha^+$ cDCs and $CD103^+$ cDCs *in vivo* (Desch *et al.*, 2011; Hildner *et al.*, 2008; Jung *et al.*, 2002).

In immature cDCs, MHC II is constitutively synthesised and loaded with peptide in endosomes, but complexes are only transiently delivered to the cell surface and are rapidly endocytosed and degraded (Wilson *et al.*, 2004). In contrast, in mature cDCs, MHC II molecules are maintained on the cell surface and *de novo* generation of MHC II-peptide complexes is downregulated (Wilson *et al.*, 2004), facilitating productive interactions with naïve T cells. pDCs do not downregulate the synthesis of new MHC II molecules following Ag exposure (Young *et al.*, 2008). This leads to constant recycling of MHC II, thus pDCs are unable to maintain MHC II-peptide complexes on their surface for prolonged periods, with detrimental effects on T cell priming (Young *et al.*, 2008). Endosomal proteases are also more active in pDCs, leading to rapid protein degradation, which prevents effective peptide loading onto MHC II (Young *et al.*, 2008). Although these characteristics mean that pDCs are less able to present exogenous Ag to $CD4^+$ T cells, they maintain the ability to present endogenous Ag on MHC I and MHC II following activation (Young *et al.*, 2008), unlike cDCs, which may highlight an important function for pDCs to present to effector T cells in peripheral tissues.

1.3.1 Signals involved in DC:T cell interaction

Although the MHC-peptide complex initially mediates interaction between an Ag-loaded DC and a naïve T cell, other signals are also required to facilitate effective T cell polarisation. The ligation of the T cell receptor (TCR) by peptide-loaded MHC provides the first stimulatory signal, which determines Ag specificity, but it is T cell co-stimulation and co-inhibition, or 'signal 2', that directs T cell function and fate (Kapsenberg, 2003). Moreover, provision of signal 1 in the absence of appropriate signal 2 can lead to the

induction of T cell anergy - unresponsiveness to Ag, with a profound defect in T cell proliferation (Bour-Jordan and Bluestone, 2009; Jenkins and Schwartz, 1987). Signal 2 is provided by the interaction of co-stimulatory/ inhibitory molecules on the surface of DCs interacting with their cognate receptor/ ligand on the surface of T cells. These molecules often co-localise with the TCR on the T cell surface, synergising with signals via the TCR that promote or inhibit T cell polarisation and activation, often these signals are involved in the control of cell survival and proliferation (Chen and Flies, 2013). The third signal that directs T cell polarisation is generally cytokines provided by DCs and most likely also from other cells within the microenvironment. However, some co-signalling interactions are also sufficient to act as signal 3 (Chen and Flies, 2013).

Co-stimulatory/inhibitory molecules primarily belong to either the immunoglobulin superfamily (IgSF) or the tumour necrosis receptor superfamily (TNFRSF). The most well known member of the IgSF is the receptor CD28, expressed on the surface of T cells (Linsley *et al.*, 1993). The activatory ligands for this receptor are CD80 and CD86 (Greenwald *et al.*, 2005; Linsley *et al.*, 1993). Other members of this family include the receptor/ ligand pair ICOS-ICOSL (Hutloff *et al.*, 1999). Co-activatory members of the TNFRSF include CD40-CD154 (CD40L) (Caux *et al.*, 1994), OX40-OX40L (Fillatreau and Gray, 2003; Ohshima *et al.*, 1997) and RANK-RANKL (Anderson *et al.*, 1997). The co-stimulatory molecules expressed by DCs are upregulated on their surface following activation and CD28 is constitutively expressed by naive T cells, helping to ensure that DC:T cell interactions lead to productive polarisation of the T cell response (Chen and Flies, 2013; Kapsenberg, 2003) (Fig. 1.5).

There are several levels of regulation of T cell co-signalling. As stated above, co-stimulatory molecules are only upregulated on the surface of DCs following their activation by Ag, or in response to some inflammatory cytokines, such as TNFα. Following TCR ligation, the co-inhibitory receptor, CTLA-4, is released from intracellular compartments and transported to the site of ligation on the T cell surface (Egen and Allison, 2002; Linsley *et al.*, 1996). Upregulation of CTLA-4 leads to the endocytosis and degradation of CD28 (Berg and Zavazava, 2008). Like CD28, CTLA-4 binds to the ligands CD80 and CD86 (Linsley *et al.*, 1996). As well as transducing inhibitory signals following ligation by CD80 and CD86, CTLA-4 can also capture these ligands, removing them from the surface of the

presenting cell and degrading them (Qureshi *et al.*, 2011), further inhibiting co-stimulatory interactions. Another co-inhibitory molecule is PD-1, a member of the IgSF (Ishida *et al.*, 1992). It has been suggested that this receptor mediates anergy of $CD8^+$ T cells (Chikuma *et al.*, 2009; Tsushima *et al.*, 2007). However, it may have a different role in controlling $CD4^+$ T cell function, facilitating the induction of inducible Tregs (iTregs) in the periphery (Francisco *et al.*, 2009; Qiao *et al.*, 2012). A role for immature DCs in the induction of $CD8^+$ T cell anergy, via their expression of PD-1 ligands and their interactions with CTLA-4, has been proposed (Probst *et al.*, 2005a). DCs have also been implicated in the PD-1-dependent induction of iTregs (Yogev *et al.*, 2012). Thus, DCs play a central role in orchestrating activatory and inhibitory responses from T cells.

1.3.2 $CD4^+$ T cell subsets

$CD4^+$ T cells are activated only by professional APCs – restricted to those cell types that express MHC II, as the TCR repertoire on $CD4^+$ T cells recognises Ag only in the context of the MHC II complex. Thus, $CD4^+$ T cells are MHC II-dependent. $CD4^+$ T cells are also MHC II-restricted as they can recognise Ag only when presented on MHC II. In 1986, distinct $CD4^+$ T cell subsets were first described, based on differential cytokine production. These subsets were termed T helper 1 (Th1) and T helper 2 (Th2) cells (Mosmann *et al.*, 1986). Since then, these subsets have been further defined, based on the cytokines, cell signals and TFs required for their differentiation, as well as their distinct functions. Furthermore, a number of other distinct $CD4^+$ T cell subsets have also been defined, including Th17 cells – named for the cytokines they produce – as well as T follicular helper (Tfh) cells and Tregs. Although it was once thought that these subsets were entirely distinct and terminally committed, there is a growing body of evidence that suggests that plasticity exists *in vivo,* allowing for some flexibility in T cell fate (Bluestone *et al.*, 2009; Zhou *et al.*, 2009) (Fig. 1.6).

Th1 cells are defined by their production of IFNγ and are the primary mediators of the adaptive response against infection by bacteria, viruses and protozoa. IFNγ produced by Th1 cells has effects on many innate effectors, including stimulating the classical activation of MΦs to kill intracellular pathogens by various mechanisms including the production of nitric oxide (Walsh and Mills, 2013). IFNγ can also stimulate the activation of anti-viral responses and can enhance the functionality of $CD8^+$ T cells during viral infection

(Platanias, 2005; Walsh and Mills, 2013). Th1 cells also provide B cell help, leading to the induction of antibody responses, particularly IgG2a, which is involved in opsonisation, complement fixation and virus neutralisation (Bluestone *et al.*, 2009). Differentiation of Th1 cells is generally dependent on IL-12, primarily from DCs (Hilkens *et al.*, 1997; Macatonia *et al.*, 1995). Polarisation of this subset can also be potentiated by IFNγ from NK cells and differentiated Th1 cells. IL-12-mediated Th1 differentiation requires signalling downstream of STAT4 (Thierfelder *et al.*, 1996), and is dependent on the induction of the TF T-bet, which potently activates transcription of the IFNγ gene (Szabo *et al.*, 2000).

Th2 cells are induced during parasitic helminth infection and produce IL-4, IL-5, IL-9, IL-10 and IL-13 (Pulendran and Artis, 2012). Th2 cells are also responsible for driving allergic responses in the airway (van Rijt and Lambrecht, 2001), and against food allergens (Berin and Mayer, 2009). The Th2 cytokines IL-4 and IL-13 stimulate alternative-activation of MΦs, which exhibit potent anti-inflammatory activity and have a role in wound healing and fibrosis (Murray and Wynn, 2011), playing an essential role in granuloma formation during helminth infection (Herbert *et al.*, 2004). IL-13 production by Th2 cells acts directly on the epithelium during enteric helminth infection, enhancing smooth-muscle contractility and epithelial cell turnover, which can aid in helminth expulsion from the intestine (Anthony *et al.*, 2007). IL-5 production by Th2 cells also activates eosinophils (EOS) and Th2 cells stimulate B cell production of IgE and IgG1 (Walsh and Mills, 2013). IL-4 signalling via STAT6 induces expression of the Th2-specific TF GATA-3 in naïve T cells, which then activates the IL-4 promoter (Kurata *et al.*, 1999; Scheinman and Avni, 2009; Zheng and Flavell, 1997). IL-2 activation of STAT5 is also integral to Th2 differentiation (Cote-Sierra *et al.*, 2004; Liao *et al.*, 2008). However, it has been postulated that a number of different cytokines that activate STAT5, including thymic stromal lymphopoeitin (TSLP) and IL-7, could also provide this crucial signal (Rochman *et al.*, 2009).

Although DCs are required for Th2 induction *in vivo* (Hammad *et al.*, 2010; Ohnmacht *et al.*, 2010; Phythian-Adams *et al.*, 2010), they do not produce IL-4 in response to Th2-polarising Ags (Jankovic *et al.*, 2004; MacDonald *et al.*, 2001), nor do they need to produce IL-4 to prime Th2 responses (Jankovic *et al.*, 2004; MacDonald *et al.*, 2002a). Similarly, DC IL-10 is not required for this process (Perona-Wright *et al.*, 2006a). However, DC expression of the co-stimulatory molecule CD40 is essential for Th2 induction

(MacDonald and Pearce, 2002), with CD40-CD154 interaction required for effective Th2 development during murine *S. mansoni* infection (MacDonald *et al.*, 2002b). Furthermore, DC expression of OX40L is necessary to maintain a sustained Th2 response (Jenkins *et al.*, 2007).

Although exogenous IL-4 is required to polarise Th2 cells *in vitro*, TCR signalling can induce low-level IL-4 production from T cells, which is sufficient to induce GATA-3 expression (Yamane *et al.*, 2005). Thus the IL-4 signal may well be cell-intrinsic, at least at the early stages of Th2 responses. It has been shown that IL-4 permeates responsive LN during helminth infection (Perona-Wright *et al.*, 2010), which may function to enhance further Th2 induction. It has also been shown that Th2 cells can be polarised *in vitro* and *in vivo* without the ability of T cells to respond to IL-4, although they are generated at a lower frequency (Jankovic *et al.*, 2000). This suggests that IL-4 may not be an essential signal for Th2 induction in all circumstances.

Recent work has suggested that other innate cells may provide the polarising early IL-4 signal. A candidate for this role is basophils, which can provide an early source of Th2 cytokines in allergic responses to peptide/protein or hapten Ags (Otsuka *et al.*, 2013; Sokol *et al.*, 2008; Yoshimoto *et al.*, 2009). Basophils may also provide an early source of IL-4 during helminth infection (Ohnmacht *et al.*, 2010; Perrigoue *et al.*, 2009). It has been suggested that basophils can present Ag and prime Th2 cells (Perrigoue *et al.*, 2009; Yoshimoto *et al.*, 2009). However, while basophils do interact with T cells in the tissue, they do not interact with Ag-specific T cells in the LN, and can only produce IL-4 in a T cell-dependent manner (Sullivan *et al.*, 2011). Further, basophils do express MHC II and can present peptide to Ag-specific T cells, but lack the machinery necessary to process and present complex Ag (Otsuka *et al.*, 2013). Moreover, a number of studies have shown that basophils are redundant during Th2 priming (Hammad *et al.*, 2010; Ohnmacht *et al.*, 2010; Phythian-Adams *et al.*, 2010; Smith *et al.*, 2012). Thus, this body of evidence discredits the idea that basophils are an essential APC population in the induction of Th2 responses, or an essential early source of polarising IL-4.

Innate lymphoid cells 2 (ILC2s), lineage-negative cells that were recently identified in the intestine and MLNs of mice infected with gastrointestinal helminths, and in the lungs of

mice in models of allergic airway responses, can also secrete Th2 cytokines during Th2 responses (Fallon *et al.*, 2006; Halim *et al.*, 2012
; Neill *et al.*, 2010; Saenz *et al.*, 2010). However, it has not been shown definitively that this population is involved in the induction of Th2 immunity, as well as at the effector stage.

Whilst it cannot be denied that a number of innate cell populations play important roles during Th2 responses, it still remains unarguable that DCs are the primary cell type required for priming Th2 cells. It is still essentially unknown what the DC-derived signals are for Th2 induction. Thus, a major goal for the field is to understand the signals provided by DCs to T cells that are integral to the initiation of Th2 polarisation.

Unlike Th1 responses, which are primarily characterised by T cell-derived IFNγ, there is a host of different Th2 cytokines, including IL-4, IL-5, IL-9, IL-10 and IL-13. It is reasonable to suggest that not all Th2 cells produce all these cytokines uniformly or simultaneously during a Th2 response. Rather, there is likely to be heterogeneity in the cytokines produced at different stages, at different sites and during different infection settings. For example, TGFβ can reprogram Th2 cells to IL-9-producing cells (Veldhoen *et al.*, 2008). It has been suggested that IL-9^{+} Th cells represent a distinct subset (Dardalhon *et al.*, 2008), but as yet no Th9-specific TF has been identified. Rather, it has been shown that IL-9 production is dependent on IRF4 (Staudt *et al.*, 2010) and PU.1 (Chang *et al.*, 2010). PU.1 is highly expressed by Th2 cells that have low-level IL-4 expression, and may enhance heterogeneity in Th2 cells by preventing GATA-3 from interacting with target loci (Chang *et al.*, 2009; Chang *et al.*, 2005). Thus, a switch to IL-9 production may reflect the plasticity of Th2 cells and their ability to respond to changes in the microenvironment.

Tfh cells are a population of T cells that provide B cell help and are required for the formation of germinal centres (Crotty, 2011). Tfh secrete IL-21 and are defined by their surface expression of ICOS, PD-1, and CXCR5 (Crotty, 2011), a chemokine receptor that facilitates localisation within the B cell zone. Tfh are integral for the formation of germinal centres and provide B cell help (in the form of IL-21, CD40-L and ICOS-L) to facilitate class-switching and plasma cell formation (Tangye *et al.*, 2013). The development of Tfh is dependent on the TF Bcl6 (Nurieva *et al.*, 2009). IL-6 and IL-21 provide signal 3 for the differentiation of this T cell subset (Eto *et al.*, 2011), whilst ICOS provides an essential

early signal to induce Bcl6 expression (Choi *et al.*, 2011b). The exact cellular sources of IL-6 and IL-21 during Tfh differentiation are not yet known, although likely include DCs, B cells and T cells (Tangye *et al.*, 2013). Studies suggest that Tfh may arise from already differentiated T effectors (Walsh and Mills, 2013). Indeed, with further evidence for the plasticity of T cell subsets, Tfh have been shown to differentiate from IL-4$^+$ Th2 cells in the reactive LN in response to helminth Ag (Glatman Zaretsky *et al.*, 2009). The primary IL-4-producing population in the dLN during helminth infection is proposed to be Tfh (King and Mohrs, 2009; Liang *et al.*, 2011). In agreement with the idea that there is heterogeneity in the cytokine profile of Th2 cells (Kelso *et al.*, 1999), it has been suggested that, during helminth infection, IL-4$^+$ T cells that do not express IL-13 are found in the B cell areas of the dLN, whilst in the tissues Th2 cells express a combination of IL-4 and IL-13, with IL-5 primarily restricted to an IL-13$^+$ subset (Liang *et al.*, 2011).

The existence of regulatory T cell populations (previously called suppressor T cells) had long been proposed, but research in this area fell out of favour due to an inability to define specific markers with which to identify this cell population (Basten and Fazekas de St Groth, 2008). Irrespective, it was known that CD4$^+$ CD25$^+$ T cells could provide protection from some inflammatory conditions, for example autoimmunity (Sakaguchi *et al.*, 1995). Scepticism about existence of this cell type changed when the Treg-specific TF Foxp3 was identified (Fontenot *et al.*, 2003; Hori *et al.*, 2003). In the absence of Foxp3, there was found to be a deficiency in the thymic development of CD4$^+$ CD25$^+$ T cells (Fontenot *et al.*, 2003) and a significant increase in self-reactive T cells in the periphery (Kim *et al.*, 2007). Thymically-derived Tregs are known as natural Tregs (nTregs), whilst regulatory T cells that develop in the periphery are described as inducible Tregs (iTregs). iTregs do not always express Foxp3, as is the case for Tr1 cells, which differentiate in response to IL-27 and produce IL-10 (Awasthi *et al.*, 2007). The maintenance of Tregs is dependent on IL-2 (Fontenot *et al.*, 2005), whilst TGFβ and retinoic acid have been shown to play a role in the differentiation of iTregs in the periphery (Chen *et al.*, 2003; Cobbold *et al.*, 2004; Coombes *et al.*, 2007). Foxp3$^+$ Tregs are induced in the steady state intestine in response to the microbiota and food Ags (Sun *et al.*, 2007), a process that is dependent on intestinal DCs (Coombes *et al.*, 2007; Sun *et al.*, 2007). Tregs in this setting play an integral role in maintaining tolerance and preventing unnecessary immune activation against innocuous Ags (Pabst and Mowat, 2012).

A key factor that led to the discovery of Th17 cells was the finding that autoimmune disease was augmented in the absence of IFNγ, which at the time was thought to be the primary mediator of these responses (Harrington *et al.*, 2005; Langrish *et al.*, 2005). Th17 cells were shown to be a separate lineage that was distinct from Th1/2 cells, with their development negatively regulated by IFNγ (Harrington *et al.*, 2005; Langrish *et al.*, 2005). Differentiation of Th17 cells is under control of the TF RORγt, and is dependent on STAT3. The cytokines TGFβ and IL-6, or IL-21, act as signal 3 for Th17 polarisation, whilst IL-23 and IL-1β enhance Th17 development and functionality (Ivanov *et al.*, 2006; Korn *et al.*, 2007; Walsh and Mills, 2013; Zhou *et al.*, 2007). It has been shown that DCs are a crucial source of IL-6 for the polarisation of Th17 cells (Perona-Wright *et al.*, 2009), it is also likely that DCs act as a source of IL-23 and IL-1β in this setting. There are a number of different sources of TGFβ *in vivo*, T cells, particularly Tregs, are one likely source (Li *et al.*, 2007; Veldhoen *et al.*, 2006), whilst T cells also likely provide IL-21, which may act in autocrine fashion (Nurieva *et al.*, 2007). Th17 cells are thought to primarily function at mucosal sites (Muranski and Restifo, 2013). As such, in the steady state they are found primarily in the lamina propria (LP) of the intestine (Ivanov *et al.*, 2006), but can readily be induced at other mucosal sites during infection (Muranski and Restifo, 2013). Consequently, Th17 cells contribute to immune responses predominantly against a variety of extracellular bacteria and fungi in the gastrointestinal tract, airway, lungs and skin (Khader *et al.*, 2009). Th17 cells produce large quantities of IL-17 and IL-22, which are taken up by epithelial cells (Khader *et al.*, 2009), leading to production of antimicrobial peptides and chemokines that attract neutrophils and other granulocytes (Kumar *et al.*, 2013) – cells that contribute to the clearance of bacterial and fungal pathogens (Khader *et al.*, 2009). However, Th17 cells are also involved in the pathogenesis of autoimmune disease in the central nervous system (Ivanov *et al.*, 2006; Langrish *et al.*, 2005), in asthma (Cosmi *et al.*, 2011), and in severe immunopathology in the gastrointestinal tract and skin – such as colitis and psoriasis (Weaver *et al.*, 2013).

Counter-regulation and antagonism are key to balancing and maintaining $CD4^+$ T cell responses. For example, T-bet suppresses transcription of the IL-4 and IL-5 genes (Szabo *et al.*, 2000), whilst GATA-3 represses IL-12 induction (Ouyang *et al.*, 1998). However, a growing body of evidence indicates a degree of plasticity within all T cell subsets, including

the ability in some contexts to co-express the subset-defining TFs and cytokines (Zhou *et al.*, 2009). For example, during viral infection, stably committed Th2 cells can be reprogrammed to become GATA-3$^+$ T-bet$^+$ cells that co-produce IL-4 and IFNγ, these cells can persist for a period of months (Hegazy *et al.*, 2010; Peine *et al.*, 2013). It has recently been shown that stable GATA-3$^+$ T-bet$^+$ T cells are found in helminth infection, including murine models of *S. mansoni* and *H. polygyrus* (Peine *et al.*, 2013). The authors suggest that these cells may function to provide both Type 1 and 2 inflammatory responses during helminth infection, whilst avoiding the severe immunopathology associated with highly Th1 or Th2 skewed responses (Peine *et al.*, 2013).

It seems that Th17 cells may display the greatest degree of plasticity of all the T cell subsets. Whilst allergic airway disease is commonly thought of as Th2-mediated, it is increasingly understood that severe, steroid-resistant asthma is associated with high levels of IL-17 and neutrophilia in the asthmatic lung (Cosmi *et al.*, 2011). In line with the fact that the allergic airway response is primarily a Th2 response, a subset of cells has been identified in mouse models and in human patients that co-express GATA-3 and RORγt, as well as Th2 and Th17 cytokines (Wang *et al.*, 2010). It appears that context has a big impact on the phenotype of Th17 cells. Whilst they remain relatively stable during acute infection with the fungus *Candida albicans*, IL-17-producing cells can be induced by IL-23 to secrete a range of other inflammatory cytokines, including IFNγ, during chronic autoimmune disease, such as experimental autoimmune encephalomyelitis (EAE) (Hirota *et al.*, 2011). Human T cells can also display this IL-17/ IFNγ double-positive phenotype *in vitro* (Boniface *et al.*, 2010). Given that both Th17 cells and Tregs depend on TGFβ for their differentiation, it is perhaps not surprising to find that there is also some crossover in these lineages. Even though Foxp3 inhibits IL-17 production (Zhou *et al.*, 2008), Foxp3$^+$ IL-17$^+$ CD4$^+$ T cells have been described both *in vitro* and *in vivo* in mouse and humans (Voo *et al.*, 2009; Yang *et al.*, 2008; Zhou *et al.*, 2008). This degree of plasticity within the CD4$^+$ T cell lineage demonstrates the complexity of the immune network and the versatility of the immune system to adapt to changes in the microenvironment.

1.4 Intestinal myeloid populations

The key feature of the intestinal immune system is its ability to maintain tolerance against dietary products and the microbiota, yet still provide protection from potential invaders

(Varol *et al.*, 2010). Intestinal mononuclear phagocytes, including DCs and MΦs, are the crucial cell populations in this setting, maintaining intestinal homeostasis, whilst acting as pathogen sentinels capable of initiating a protective immune response (Coombes and Powrie, 2008; Varol *et al.*, 2010). Studying intestinal myeloid populations presents significant challenges, as isolation of cells from the intestinal tissue is difficult, particularly under inflammatory and infection conditions. Additionally, the overlapping expression profiles of the surface markers on intestinal DCs and MΦs has led to confusion and conflicting reports on these distinct cell populations. Increasingly, however, a consensus is emerging and our understanding of these cells and their role in immune processes has improved significantly in recent times.

1.4.1 Intestinal DCs

Intestinal DCs are located in organised lymphoid tissues, such as Peyer's Patches (PPs) and isolated lymphoid follicles (ILFs), as well as distributed throughout the villous lamina propria (LP) (Coombes and Powrie, 2008; Johansson-Lindbom *et al.*, 2005; Scott *et al.*, 2011). DCs at these sites are further subdivided into specific subsets, based on their surface expression of CD11b and the integrin CD103 (αEβ7) (Bogunovic *et al.*, 2009; Varol *et al.*, 2010) (Fig. 1.7). Intestinal DCs constantly monitor the mucosa, capturing Ag and constitutively trafficking to the MLNs in the steady state (Cerovic *et al.*, 2012). Interaction of T cells with intestinal DCs in the MLNs leads to the generation of gut-tropic T cells that upregulate the gut-homing markers CCR9 and $\alpha_4\beta_7$ (Jaensson *et al.*, 2008; Johansson-Lindbom *et al.*, 2005), a process that is dependent on the ability of intestinal DCs to metabolise dietary retinoids to retinoic acid (RA) (Iwata *et al.*, 2004). It was originally reported that $CD103^+$ DCs are uniquely able to generate a gut-tropic T cell population (Jaensson *et al.*, 2008). However, recent work has shown that $CD103^-$ $CD11b^+$ DCs migrating from the intestine also have the capacity to confer gut tropism on differentiating T cells (Cerovic *et al.*, 2012). This discrepancy may reflect the fact that Cerovic *et al*. restricted their analysis to the DC subsets migrating in the intestinal lymph, whilst previous studies tested the DC populations found in the MLNs, PPs or LP (Jaensson *et al.*, 2008; Johansson-Lindbom *et al.*, 2005). Presentation of innocuous Ag, such as dietary products or commensal bacteria, by intestinal DCs in the absence of an inflammatory stimulus does not lead to the initiation of an immune response but induces a

state of tolerance and immune unresponsiveness (Pabst and Mowat, 2012; Scott *et al.*, 2011; Worbs *et al.*, 2006).

Intestinal DCs, particularly $CD103^+$ DCs, display a potent capacity to induce the generation of $Foxp3^+$ Tregs in the steady state (Coombes *et al.*, 2007), which may help to maintain a tolerogenic environment (Hadis *et al.*, 2011). RA acts as a co-factor for the TGFβ-mediated generation of $Foxp3^+$ Tregs by intestinal DCs (Coombes *et al.*, 2007). Intestinal DCs themselves may well provide a source of TGFβ for Treg induction, as $CD103^+$ LP DCs express high levels of the TGFβ gene (Coombes *et al.*, 2007). $CD103^+$ MLN and LP DCs also express the αvβ8 integrin (Worthington *et al.*, 2011), which converts the latent form of TGFβ to its active state (Mu *et al.*, 2002). In the absence of αvβ8 on intestinal DCs, the normal population of intestinal Tregs is not maintained, leading to severe pathology (Travis *et al.*, 2007). These properties are not shared by $CD103^+$ DCs in other tissues, and seem to be imprinted on DCs by the gut itself (Scott *et al.*, 2011). Intestinal epithelial cells may play a role in this, promoting a tolerogenic phenotype in gut DCs via their production of TGFβ, RA and TSLP (Iliev *et al.*, 2009a; Iliev *et al.*, 2009b). It has also been postulated that constitutive exposure to dietary metabolites and commensal-derived microbial components contributes to the conditioning of DCs and promotes a tolerogenic microenvironment in the steady state gastrointestinal tract (Scott *et al.*, 2011).

The intestinal DC subsets have distinct transcriptional requirements for their development, with the differentiation of the $CD103^+$ $CD11b^-$ LP population (like the $CD8\alpha^+$ splenic cDC subset) dependent on Id2, IRF8 and Batf3 (Edelson *et al.*, 2010; Ginhoux *et al.*, 2009). The differentiation of the $CD103^+$ $CD11b^+$ LP DC population is dependent on IRF4 (Persson *et al.*, 2013). This subset of LP DCs also depends on Notch2 signalling for their development (Satpathy *et al.*, 2013). As in other tissues, GM-CSF is required for $CD103^+$ LP DC homeostasis (Greter *et al.*, 2012) and Flt3-L is required for their differentiation (Bogunovic *et al.*, 2009; Ginhoux *et al.*, 2009). Like other $CD11b^+$ DC subsets, the $CD103^-$ $CD11b^+$ LP DC population is also deficient in CD11c-specific, IRF4-deficient animals in the steady state, although this subset is restored under inflammatory conditions (Persson *et al.*, 2013).

Although $CD103^+$ LP DC subsets are well known for their ability to polarise $Foxp3^+$ Tregs, CD11c-specific IRF4-deficient mice have a selective deficiency in the intestinal Th17 population, with further analysis demonstrating that the $CD103^+$ $CD11b^+$ LP DC subset is an essential source of IL-6 that is required for Th17 development in the intestine (Persson *et al.*, 2013). This finding demonstrates that the intestinal DC subsets do not have discrete functions in controlling the intestinal immune system. Indeed, despite early studies suggesting that $CD103^+$ DCs are the only LP DC subset to migrate to the MLN and polarise T cells (Schulz *et al.*, 2009), comprehensive characterisation of cells in intestinal lymph highlights that the $CD103^+$ $CD11b^+$, $CD103^+$ $CD11b^-$ and $CD103^-$ $CD11b^{+/-}$ LP DC subsets all migrate, and can be present in similar proportions to those found in intestinal tissue (Cerovic *et al.*, 2012). Further to this, all of the LP DC subsets show some capacity to stimulate T cell proliferation and imprint gut tropism (Cerovic *et al.*, 2012) (Fig. 1.9).

A glaring omission in the characterisation of the LP DC subsets is any level of understanding of the role of these cells in active infection. This is an area of ongoing study for many groups. A very recent study has demonstrated that Notch2-dependent $CD103^+$ $CD11b^+$ LP DCs are an essential source of IL-23 during infection with the pathogenic bacteria, *Citrobacter rodentium* (Satpathy *et al.*, 2013). Production of IL-23 by $CD103^+$ $CD11b^+$ DCs is essential for host survival. The roles that different intestinal DC subsets may play in the induction of Th2 responses is unknown.

1.4.2 Macrophages in the intestine

Many studies mistakenly characterise LP MΦs as a DC population, due to their mid- to high-level expression of the DC-associated marker CD11c (Bogunovic *et al.*, 2009; Ginhoux *et al.*, 2009; Niess and Adler, 2010; Niess *et al.*, 2005). Initially, a population of $CX3CR1^+$ DCs was identified in the LP (Niess *et al.*, 2005). Expression of CX3CR1 (the fractalkine receptor) allows this cell population to interact with epithelial cells expressing CX3CL1 and to form dendrites, which traverse the epithelial layer and sample Ag from the lumen (Niess *et al.*, 2005). However, further characterisation of this population revealed that $CX3CR1^+$ myeloid cells are in fact MΦs, not DCs. This was demonstrated by the fact that the LP MΦ population has a distinct ontogeny to LP DC subsets, differentiating from monocytes in response to M-CSF, whilst the LP DCs develop from CDPs in a Flt3-L dependent manner (Bogunovic *et al.*, 2009; Ginhoux *et al.*, 2009; Varol *et al.*, 2009).

Further, LP DC subsets express the cDC TF Zbtb46, whilst intestinal MΦs do not (Satpathy *et al.*, 2012a). Importantly, CX3CR1^{+} MΦs do not migrate to the MLN and display poor T cell priming ability (Cerovic *et al.*, 2012; Schulz *et al.*, 2009). However, to add to the confusion surrounding this issue, CX3CR1 expression is not restricted to LP MΦs alone, as some LP DCs can also express this marker, although at much lower levels (Bain *et al.*, 2012; Cerovic *et al.*, 2012). Thus, CD64, or FcγR1, is increasingly being used as an alternative marker to CX3CR1 to differentiate between LP DCs and MΦs, as it appears to be expressed selectively by the MΦ population (Bain *et al.*, 2012; De Calisto *et al.*, 2012; Tamoutounour *et al.*, 2012).

What is the function of LP MΦs, which do not migrate (at least in the steady state), and therefore do not contribute to T cell priming? As highlighted above, LP MΦs are capable of sampling Ag from the lumen (Niess and Reinecker, 2005), perhaps providing a source of Ag for LP DCs to present to T cells in the MLN (Schulz and Pabst, 2013; Varol *et al.*, 2010), for tolerance induction in the steady state or to aid the induction of the adaptive response during active infection (Niess and Reinecker, 2005). Human intestinal MΦs are highly phagocytic (Smythies *et al.*, 2005) and CX3CR1^{+} MΦs in mouse are found adjacent to the intestinal epithelium, whilst LP DCs reside in the core of the villus (Schulz *et al.*, 2009). These characteristics are all in agreement with the Ag-sampling function of LP MΦs. It has also been suggested that these cells may function to take up and destroy free Ag, before it is capable of activating an unnecessary immune response (Persson *et al.*, 2010).

Although the initial generation of gut-tropic Tregs occurs in the MLNs, under the control of intestinal DCs, their full differentiation depends on their migration to the intestinal mucosa, where they undergo further expansion under the influence of IL-10-producing CX3CR1^{+} MΦs (Hadis *et al.*, 2011), indicating an important role for LP-resident MΦs in maintaining immune tolerance in the steady state. The production of IL-10 by LP MΦs depends on their expression of CX3CR1 (Hadis *et al.*, 2011), further demonstrating the importance of the epithelial cell layer in the induction and maintenance of immune tolerance. Similarly to LP DC subsets, intestinal MΦs have a tolerogenic phenotype in the steady state and are refractory to TLR stimulation (Bain *et al.*, 2012; Denning *et al.*, 2007; Monteleone *et al.*, 2008). Whether this phenotype is maintained during infection remains to be determined.

However, gene expression analysis of LP-resident MΦs demonstrates that this cell population displays a non-inflammatory profile in a DSS-dependent model of colitis (Zigmond *et al.*, 2012).

In the steady state, the LP MΦ population is renewed by Ly6C^{hi} CX3CR1^{lo} monocytes recruited from the blood (Bain *et al.*, 2012; Varol *et al.*, 2009). However, under inflammatory conditions this process is not maintained and inflammatory monocytes accumulate (Bain *et al.*, 2012; Zigmond *et al.*, 2012). This occurs both in models of colitis (Bain *et al.*, 2012; Platt *et al.*, 2010; Tamoutounour *et al.*, 2012; Zigmond *et al.*, 2012) and during infection with the parasitic protozoan species *Toxoplasma gondii* (Grainger *et al.*, 2013). Unlike steady state LP MΦs, inflammatory monocytes in the colitic large intestine are responsive to TLR stimulation, producing higher levels of inflammatory mediators, such as IL-6, TNFα and nitric oxide (Bain *et al.*, 2012; Tamoutounour *et al.*, 2012; Zigmond *et al.*, 2012). Tamoutounour *et al*. (2012) demonstrated that inflammatory monocytes also accumulate in the MLNs and can polarise Th1 IFNγ^{+} cells. The authors suggested that inflammatory monocytes could orchestrate the inflammatory T cell response in this setting as inflammation develops in CCR7-deficient animals, indicating that migrating DCs do not play a role (Tamoutounour *et al.*, 2012). In contrast, Zigmond *et al*. (2012) demonstrated that inflammatory monocytes acquire expression of the DC TF Zbtb46, are capable of migrating in intestinal lymph, and can stimulate T cell proliferation. The discrepancies in the findings of these groups may relate to the use of different colitis models: the T cell transfer model (Tamoutounour *et al.*, 2012) vs. DSS-induced inflammation (Zigmond *et al.*, 2012). Nonetheless, both of these studies propose a role for monocytes in priming T cells under inflammatory conditions, suggesting that infiltrating innate cell populations can directly control the adaptive immune response. In contrast, in *T. gondii* infection, infiltrating inflammatory monocytes in the small intestinal LP (SI LP) regulate the expansion of infiltrating neutrophils and dampen immunopathology in the gut (Grainger *et al.*, 2013), suggesting that there is diversity in the impact of monocytes on intestinal inflammation depending on context.

Yet to be addressed is the role of these cells, or LP-resident myeloid cell populations, in the immune response against parasitic helminth infection in the intestine, a unique setting where AAMΦs are essential (Herbert *et al.*, 2004; Herbert *et al.*, 2010). In *Litomosoides*

sigmodontis infection (where the adult worms reside in the pleural cavity (Le Goff *et al.*, 2000), resident MΦs become alternatively-activated. Further, an increase in MΦ numbers at the infection site is due to the proliferation of the resident population, under control of IL-4 signalling (Jenkins *et al.*, 2011). In this setting, there is only a very minor influx of inflammatory monocytes, relatively late in infection. Thus the role of myeloid cells, including their proliferation/recruitment, activation and interaction with other cell types, may be entirely different during Th2 infections and inflammation than even the limited descriptions that currently exist in Th1/17 infection settings.

1.5 Type I IFN

Although IFN-I provides the first line of defence against viral infection, this family of cytokines has broad and wide-reaching influence on a range of immune mechanisms, and is capable of activating the expression of several hundred to over a thousand different genes (de Veer *et al.*, 2001; Der *et al.*, 1998; Lanford *et al.*, 2006). Further, IFN-I plays a unique role in the immune system, due to its ability to influence both innate and adaptive immunity.

1.5.1 Activation and signalling downstream of IFNAR

IFN-I is a large cytokine family in humans and mice, comprising 12 IFNα subtypes (IFNα1-13, IFNα1 and IFNα13 are identical), IFNβ, IFNε, IFNκ and IFNω (Piehler *et al.*, 2012). Despite the size of this family, all subtypes bind to a common receptor – IFNAR, a heterodimer comprised of IFNAR1 and IFNAR2 subunits (Platanias, 2005). IFNAR1 has low affinity, and IFNAR2 high affinity, for their ligands (de Weerd and Nguyen, 2012). The IFNα subtypes display 70-80% sequence homology and share around 35% homology with IFNβ (Genin *et al.*, 2009). The affinity each IFN-I subtype has for its receptor, the stability of the ternary complex formed by ligand binding, and the number of receptors present on the cell surface, all impact on the gene expression profile that follows IFNAR ligation (de Weerd and Nguyen, 2012; Kalie *et al.*, 2008; Levin *et al.*, 2011; Piehler *et al.*, 2012). The genes activated by IFN-I also depend on the range of IFN-I subtypes that are present, as distinct Ags stimulate production of a specific suite of IFN-I family members (Genin *et al.*, 2009; Hillyer *et al.*, 2012).

IFNAR2 has three isoforms, transcribed from the same gene by alternative splicing (de Weerd and Nguyen, 2012). The most well characterised isoform, IFNAR2c, exists in a long transmembrane form with a complete intracellular domain (de Weerd and Nguyen, 2012). This isoform is required for optimal IFNAR signalling and induction of anti-viral responses (Cohen *et al.*, 1995). IFNAR2b is a short transmembrane form, which lacks the intracellular domain, and there is some suggestion that this form may act as a dominant negative, or decoy, receptor (Gazziola *et al.*, 2005; Pfeffer *et al.*, 1997). IFNAR2a is a soluble isoform, it has been shown that it can inhibit binding of IFN-I to IFNAR1-IFNAR2c, and may antagonize downstream signalling, however, this has so far only been documented *in vitro* (Hardy *et al.*, 2001).

Signalling downstream of IFNAR is dependent on Janus activated kinase (JAK) and STAT activity. IFNAR1 is constitutively associated with the JAK tyrosine kinase 2 (TYK2), whilst IFNAR2 associates with JAK1 (de Weerd and Nguyen, 2012; Platanias, 2005). The receptor-associated JAKs become autophosphorylated and activated following ligand-dependent rearrangement and dimerisation of the receptor subunits (Platanias, 2005). The activated receptor-associated JAKs then phosphorylate STATs, which form homo- or heterodimers and translocate to the nucleus to regulate gene transcription, binding to specific sites in the promoters of ISGs (de Weerd and Nguyen, 2012; Piehler *et al.*, 2012). The STATs activated by IFN-I include STAT1, STAT2, STAT3 and STAT5 (Platanias, 2005).

Following ligation of IFNAR, a homodimer of STAT1 and STAT2 can form a complex with IRF9 called IFN-stimulated gene factor 3 (ISGF3), which binds to IFN-stimulated response elements (ISREs) in the promoter regions of ISGs (Platanias, 2005) (Fig. 1.8). This transcriptional activator is particularly important in potentiating the IFN-I signal, as it stimulates the transcription of the IRF7 gene, itself an essential TF for the induction of the full family of IFNα subtypes (Marie *et al.*, 1998; Sato *et al.*, 1998). STAT complexes that do not recruit IRF9 bind to IFNγ-activated site (GAS) enhancer elements in gene promoters, activation of which can be induced by both IFN-I and IFNγ (Gonzalez-Navajas *et al.*, 2012; Platanias, 2005). Some genes have only ISREs or only GAS elements, whilst others have both elements, thus a combination of different STAT-containing complexes may be required for optimal transcription of a particular gene.

IFNAR ultilises a number of other signalling pathways to alter gene transcription and cellular function, including MAPK and activation of a CRK-dependent pathway. The adaptor protein CRK-like (CRKL) associates with TYK2 and becomes phosphorylated following ligation of IFNAR (Ahmad *et al.*, 1997). Activated CRKL then forms a complex with IFN-I-activated STAT5, this complex translocates to the nucleus and binds GAS elements to activate ISGs (Fish *et al.*, 1999) (Fig. 1.9a). Phosphorylation of CRKL also leads to the activation of C3G – a guanine-nucleotide exchange factor (GEF), which faciliatates the activation of the GTPase Rap1 (Raaijmakers and Bos, 2009) (see Fig. 1.9a). Rap1 and its downstream effectors are major players in the regulation of integrin activation, and therefore cell adhesion, migration and cell-cell interactions (Boettner and Van Aelst, 2009; Ebisuno *et al.*, 2010; Hogg *et al.*, 2011).

Of all the MAPK pathways, it seems that p38 is the most important for mediation of IFN-I-activated signals (Platanias, 2005). Phosphorylation of p38 leads to its activation; this process is dependent on a three-kinase relay system, which involves MAPKKK phosphorylating and activating MAPKK, which lastly activates MAPK, itself also a kinase (Zhang and Dong, 2005) (Fig. 1.9b). Activated p38 subsequently regulates activation of multiple downstream effectors, including MAPK-activated protein kinase 2 (MAPKAPK2), MAPKAPK3, mitogen- and stress-activated kinase 1 (MSK1) and MAPK-interacting protein kinase 1 (MNK1) (Platanias, 2005).

1.5.2 DC sources of IFN-I

pDCs are often described as “professional” IFN-I producers because of their ability to respond to viral challenge, particularly via TLR7/9, with rapid and robust IFN-I production (Reizis *et al.*, 2011). Other cell types, including cDCs, respond to viral infection with a much lower level, but more prolonged, IFN-I signal. This raises the question of why we need a dedicated cell type for an IFN-I response. The rapid yet transient IFN-I signal from pDCs may provide a necessary stimulus to activate an immune response, whilst avoiding excessive uncontrolled inflammation that could prove damaging in the long-term. This is advantageous in viral infection because many viruses need to be detected and controlled within a matter of hours to prevent dangerous cell damage or rapid replication that is difficult to curtail, and can lead to T cell exhaustion (Swiecki *et al.*, 2010). The role of pDCs

as a source of IFN-I is not restricted to antiviral responses, however, as they can also respond to self nucleic acid ligands that are released following tissue injury. For example, pDC production of IFN-I is key to orchestrating immune activation and wound healing following damage to the skin (Gregorio *et al.*, 2010). Thus, pDCs function primarily as immune sentinels and IFN-I producers, with antigen-presentation to T cells a secondary concern.

1.5.3 Impact of IFN-I on DC function

A number of studies have suggested a role for IFN-I in controlling DC turnover and survival *in vivo*. Purified $CD11c^+$ cells from the spleen of IFNAR-deficient mice are more resistant to apoptosis *in vitro*, whilst systemic IFNα administration augments DC turnover (Mattei *et al.*, 2009). These findings suggest that IFN-I may promote DC apoptosis. Indeed, IFN-I can induce expression of pro-apoptotic molecules and caspases by BMDCs and splenic cDCs (Fuertes Marraco *et al.*, 2011; Mattei *et al.*, 2009; Yen and Ganea, 2009), whilst systemic pI:C and LCMV infection – both potent stimulators of IFN-I – lead to a significant depletion of cDCs in the spleen (Fuertes Marraco *et al.*, 2011). During viral infection, pDCs also upregulate caspases and pro-apoptotic proteins, undergoing IFNAR-dependent cell death (Swiecki *et al.*, 2010). This enhancement of pDC turnover may serve to prevent excessive IFN-I-mediated cell activation; similarly, a reduction in cDC numbers would also help to curtail an immune response. Thus, the interplay between IFN-I and DCs is complex, and the effects of IFN-I on DC function multi-faceted.

Whilst pDC IFN-I acts as a short, sharp signal to cells in the surrounding microenvironment, cDCs produce low-level IFN-I that acts as an autocrine stimulus to enhance cDC function (Montoya *et al.*, 2002). For example, optimal IL-12 production by GMDCs and human mo-DCs following TLR stimulation depends on an endogenous IFN-I signal (Gautier *et al.*, 2005). In the absence of functional IFNAR, or following IFNAR blockade, the ability of DCs to produce both IFN-I and IL-12 is severely compromised. This effect was seen in DCs exposed to pI:C and R848, which stimulate TLR3/ cytosolic nucleic acid sensors, and TLR7/8 respectively, but also following stimulation with LPS alone or in combination with pI:C/ R848 (Gautier *et al.*, 2005). In the absence of IFNAR signalling *in vivo,* splenic or LN cDCs also display a more muted level of surface activation in response

to systemic pI:C treatment, and their ability to prime naïve $CD4^+$ T cells is severely impaired (Kurche *et al.*, 2012; Longhi *et al.*, 2009).

1.5.4 DC IFN-I and activation of immune responses against pathogens

As suggested by the work described above studying cDC responses to LPS, cDC IFN-I also has a role to play in immune activation against bacteria infection. IFN-I signalling is required for protection against infection by a range of bacterial species, including group B streptococci (GBS), pneumococci and *Escherichia coli* (Mancuso *et al.*, 2009). In the case of GBS, cDCs are the primary source of IFN-I, utilising a distinct TLR7/ IRF1-dependent pathway to initiate IFN-I production (Mancuso *et al.*, 2009). The importance of cDC-derived IFN-I in this setting was demonstrated in TLR7-deficient and IRF1-deficient animals, which are highly susceptible to GBS infection (Mancuso *et al.*, 2009). GMDCs also produce IFNβ following exposure to fungal pathogens, including *Candida* species and *Saccharomyces cerevisiae* (Biondo *et al.*, 2011; Bourgeois *et al.*, 2011; Del Fresno *et al.*, 2013). In the absence of the IFN-I response, mice infected with *C. albicans* are unable to control fungal growth and are more susceptible to infection than wild-type (WT) animals (Biondo *et al.*, 2011; Del Fresno *et al.*, 2013). This susceptibility is associated with a reduction in neutrophil and $CD4^+$/$CD8^+$ T cell infiltration (Del Fresno *et al.*, 2013).

A systemic IFN-I signal is required for DC mobilisation during *Plasmodium* infection (Guermonprez *et al.*, 2013). This involves a complex pathway where IFN-I regulates uric acid production by infected erythrocytes, these uric acid crystals trigger Flt3-L release from mast cells, and Flt3-L facilitates the expansion of the $CD8\alpha^+$ cDC population, which greatly enhances the magnitude of the $CD8^+$ T cell response. Cross-presentation by $CD8\alpha^+$ cDCs is essential for $CD8^+$ T cell priming during viral infection and in anti-tumour responses (Hildner *et al.*, 2008). The ability of $CD8\alpha^+$ cDCs to cross-present and activate $CD8^+$ T cells depends on their ability to respond to IFN-I, at least in the context of viral infection (Le Bon *et al.*, 2003; Pinto *et al.*, 2011), and in immune rejection of tumours (Diamond *et al.*, 2011; Fuertes *et al.*, 2011), demonstrating the importance of IFN-I for optimal DC function in active immune responses.

1.6 Helminth infection

1.6.1 Schistosome infection

200 million people worldwide, primarily in tropical and subtropical regions, are chronically infected with schistosome parasites (Wilson *et al.*, 2007). Schistosome infection is predominantly a problem for developing countries in the tropics, however, the increase in foreign travel to these areas and the fact that this is a water-borne disease, mean that more Westerners are also being exposed to infection. Schistosomes are intravascular trematodes and are unusual helminths because of the fact that they can cause severe disease, which can be lethal (Gryseels *et al.*, 2006). Three parasite species cause most cases of human schistosomiasis: *Schistosoma mansoni, Schistosoma haematobium* and *Schistosoma japonicum* (Dunne and Cooke, 2005; Gryseels *et al.*, 2006). It is estimated that around 280,000 deaths a year in sub-Saharan Africa are due to *S. mansoni* and *S. haematobium* infection (Pearce and MacDonald, 2002).

Schistosoma cercariae are shed by the intermediate host, freshwater snails, and can survive for up to 72h in the water, seeking the skin of a suitable definitive host (Gryseels *et al.*, 2006). Following penetration of the skin, larvae migrate in the blood via the lungs to the perivesical (*S. haematobium*) or mesenteric (other species) vasculature, where they develop into adult worms and pair-up (Wilson and Barnes, 1977). Around 30-32 days after infection, female worms begin to release eggs (Pellegrino *et al.*, 1962), and it is estimated that the female worms of the African species (*S. mansoni* and *S. haematobium*) lay hundreds of eggs a day (Gryseels *et al.*, 2006; Moore and Sandground, 1956; Wilson *et al.*, 2007). Following worm pairing, female worms begin to express a TGFβ family member, *S. mansoni* Inhibin/Activin (SmInAct), in their reproductive tract (Freitas *et al.*, 2007). This molecule is also expressed in eggs laid by female worms, and is essential for development of the parasite embryo and reproductive success of the adult worm (Freitas *et al.*, 2007). It takes approximately 6 days for *S. mansoni* eggs to fully mature once they have been laid (Pellegrino *et al.*, 1962), and from around day 42 of infection eggs are detectable in the faeces (Moore and Sandground, 1956). *S. mansoni* eggs must pass from the vasculature into the intestinal lumen in order to exit the host (Pearce and MacDonald, 2002), whilst *S. haematobium* eggs traverse the bladder wall and are excreted in the urine (Gryseels *et al.*, 2006). *S. mansoni* eggs are also swept from the vasculature into the liver by the flow of blood. Eggs trapped in the liver cause extreme pathology and can lead to gross periportal

fibrosis and hypertension (Wilson *et al.*, 2007). Severe morbidity is associated with the egg-specific Type 2 inflammation and granulomas in the gut and liver tissues (*S. mansoni*) or the urogenital tract (*S. haematobium*) (Dunne and Cooke, 2005).

The fibrotic response in *S. mansoni* infection is initiated by and dependent on $CD4^+$ T cells (Phillips *et al.*, 1977), leading to the generation of collagen-rich granulomas that encapsulate eggs trapped in the tissue, sequestering egg products (Pearce and MacDonald, 2002). Granulomas are primarily made up of immune cells, including $CD4^+$ T cells, EOS and MΦs (Pearce and MacDonald, 2002; Weinstock and Boros, 1983). Around the onset of egg deposition a strongly Th2-polarised immune response develops, and it is Ags released by the eggs themselves that initiate this response (Grzych *et al.*, 1991; Pearce *et al.*, 1991). In the absence of the Th2 cytokines IL-4 and IL-13, the granulomatous response is curtailed in the liver and the intestine, leading to severe Th1-skewed inflammation in the intestine, a breakdown of intestinal barrier function and an increase in mortality (Fallon *et al.*, 2000). In mice that are deficient in these Th2 cytokines severe haemorrhagic lesions develop in the mucosa of the small intestine (Brunet *et al.*, 1997; Fallon *et al.*, 2000), which may allow the translocation of commensal bacteria from the intestinal lumen, with death likely caused by cytokine-induced septic shock (Fallon *et al.*, 2000; Wilson *et al.*, 2007). IL-4 is also required to protect against severe liver damage (Brunet *et al.*, 1999; Brunet *et al.*, 1997), in the absence of the granulomatous response, there is toxic leakage of egg components into the tissue, this leads to necrosis, as hepatocytes are susceptible to egg-derived toxins (Pearce and MacDonald, 2002; Wynn *et al.*, 2004). The importance of IL-4 and IL-13 in the development of granulomas during *S. mansoni* infection is further demonstrated by the fact that this response is also curtailed in the absence of the IL-4 receptor α subunit (a component of the IL-4 and IL-13 receptor)(Jankovic *et al.*, 1999) and STAT6-deficient mice (activated downstream of IL-4 and IL-13 receptors)(Kaplan *et al.*, 1998). The Th2 response not only prevents the development of this severe pathology, but is also required for the successful transit of eggs through the intestinal tissue (Fallon *et al.*, 2000). In IL-4-deficient and IL-4/IL-13-deficient animals, eggs cannot successfully traverse the intestine and become trapped in the intestinal wall, leading to a dramatic reduction in the number of eggs present in the faeces (Fallon *et al.*, 2000). This demonstrates the importance of a functional Th2 granulomatous response not only for the protection of the host, but also to mediate the

successful trafficking of parasite eggs out of the host. A functional T cell response is also required for the generation of antibodies that protect from the hepatotoxicity of parasite excretory/ secretory products (Doenhoff *et al.*, 1981), such as omega-1 (Dunne *et al.*, 1981), a highly immunostimulatroy molecule secreted by *S. mansoni* eggs (Everts *et al.*, 2012; Everts *et al.*, 2009; Steinfelder *et al.*, 2009).

Similarly to *Il4*$^{-/-}$/*Il13*$^{-/-}$ mice, study of *S. mansoni* infected LysM^{Cre}IL-4Rα$^{-/flox}$ mice, which are unable to respond to IL-4/13 in the neutrophil and macrophage compartment, display an increased Th1 response, and severe intestinal damage leading to bacterial translocation and sepsis (Herbert *et al.*, 2004). This phenotype is not dependent on neutrophils, rather demonstrating the importance of alternatively-activated MΦs (AAMΦs) in the intestinal response (Herbert *et al.*, 2004). This is in agreement with an earlier finding that the majority of cells within intestinal granulomas are MΦs (Weinstock and Boros, 1983).

These studies in mice that are deficient in Th2 responses demonstrate that Th2 immune activation is host protective and is required for wound healing following the damage caused to organs by egg transit. However, there is no real evidence that this response is able to kill or expel schistosome worms, whereas in intestinal helminth infection, the Th2 response is more effective in bringing about parasite expulsion (Anthony *et al.*, 2007). This suggests that the localisation of schistosomes is key to their longevity and to the failure of the Th2 response to successfully target such intravascular parasites for destruction or removal.

The peak of the Th2 response during murine *S. mansoni* infection is around d56 of infection (Pearce and MacDonald, 2002). From d84 onwards this acute Th2 response gives way to a more regulatory phenotype (Dunne and Cooke, 2005). This timepoint is also characterised by T cell exhaustion, whilst Th2 cell numbers are not reduced, these cells display an inability to proliferate (Taylor *et al.*, 2009). The regulatory phenotype in chronically infected mice is characterised by downmodulation of the granulomatous response in the liver and the colon, but not the small intestine (Weinstock and Boros, 1981). Downmodulation of this response in the colon is associated with a reduction in $CD4^+$ T cell infiltration and reduced collagen deposition in granulomas, likely caused by a significant decrease in Th2 cytokines in the tissue environment (Turner *et al.*, 2011).

Regulation of granuloma formation in the colon is mediated by Tregs and an increase in TGFβ (Turner *et al.*, 2011). Tregs also prevent the development of severe pathology in the liver (Layland *et al.*, 2007), by both IL-10 dependent and independent mechanisms (Baumgart *et al.*, 2006; Hesse *et al.*, 2004). However, IL-10 production is not restricted to Tregs as $CD4^+$ T effectors and innate cells are important sources of IL-10 during *S. mansoni* infection (Dewals *et al.*, 2010; Hesse *et al.*, 2004). Whilst IL-10 and TGFβ are essential regulators of the immune response in the liver (Herbert *et al.*, 2008), it has been demonstrated that Arginase-I production by AAMΦs is a primary regulatory factor in the small intestine, and is essential for preventing excessive tissue injury (Herbert *et al.*, 2010; Pesce *et al.*, 2009).

Many people suffering from chronic schistosomiasis also display downmodulation of immune mechanisms, demonstrated by a reduction in lymphocyte responsiveness to Ags from different schistosome lifecycle stages in individuals chronically infected with *S. mansoni* (Ottesen *et al.*, 1978). The schistosome-specific IL-5 and IFNγ responses of peripheral blood mononuclear cells (PBMCs) from individuals reinfected with *S. haematobium* after drug treatment are also reduced compared to uninfected subjects (Grogan *et al.*, 1998), whilst infected individuals living in an area where *S. mansoni* is endemic also display a reduction in IFNγ responses compared to “endemic normals” (those who are egg-negative) (Viana *et al.*, 1994). PBMCs from individuals who are chronically infected with *S. mansoni* also display a reduction in their ability to produce IFNγ (de Jesus *et al.*, 1993; Zwingenberger *et al.*, 1989). In agreement with this regulatory phenotype, PBMCs purified from *S. haematobium* infected children display increased schistosome-specific IL-10 production (Meurs *et al.*, 2011; van den Biggelaar *et al.*, 2000). However, the pro-inflammatory cytokine response to TLR2 ligands is enhanced, suggesting that suppression of the host immune system in schistosome-infected individuals is not total (Meurs *et al.*, 2011). As such, systemic IL-4, IL-10 and IL-13 levels are reduced in *S. haematobium* infected individuals, however IL-2 and IFNγ in the serum of these subjects is elevated compared to those that are egg-negative (Milner *et al.*, 2010).

In agreement with findings from mouse models of schistosome infection (Brunet *et al.*, 1997; Fallon *et al.*, 2000), asymptomatic schistosomiasis in humans is associated with

elevated IL-5, whilst those suffering from severe hepatosplenic disease display high levels of IFNγ, TNFα and nitric oxide (Mwatha *et al.*, 1998).

Despite the downregulation of immune function in chronically infected individuals, immunity to schistosome infection does eventually develop. Children in schistosome endemic areas are heavily infected, whilst adults living in the same areas show low or no detectable signs of infection (Butterworth *et al.*, 1988; Chandiwana and Woolhouse, 1991). This is the case despite equal exposure of adults and children to water heavily infested with schistosome larvae (Kabatereine *et al.*, 1999). The development of immunity occurs at an earlier age in individuals living in areas where transmission is high, suggesting that resistance to reinfection develops after a certain number of transmissions (Woolhouse *et al.*, 1991). However, there also seems to be adult-intrinsic immunity to schistosomes that children lack. This is illustrated by the development of acquired immunity within 3 years of adults being exposed to schistosomes, whilst children under the same conditions remain susceptible to reinfection (Naus *et al.*, 1999; Stelma *et al.*, 1993).

The exact nature of the protective immune response has not been identified. However, enhanced antibody responses, particularly IgE and IgG1 (Black *et al.*, 2010; Demeure *et al.*, 1993; Mutapi *et al.*, 1998; Pinot de Moira *et al.*, 2010), have been associated with resistance to reinfection (Hagan *et al.*, 1991; Mutapi *et al.*, 2008). It has been suggested that this protective antibody response is stimulated by the death of adult worms, providing an antigenic stimulus that generates antibodies to inhibit worm fecundity (Mitchell *et al.*, 2012; Woolhouse and Hagan, 1999). The average lifespan of worms is around 5-10 years (Fulford *et al.*, 1995); the decline in disease prevalence in adults coincides with the time at which worms acquired in childhood would naturally begin to die (Black *et al.*, 2010). By treating individuals with the anti-schistosome drug praziquantel, the protective immune response is boosted by drug-induced worm killing and resistance develops more quickly (Black *et al.*, 2010; Mutapi *et al.*, 1998).

1.6.2 Immune recognition of schistosome eggs

Egg deposition during *S. mansoni* infection stimulates a switch from a mixed Th1-Th2 immune response to a dominant Th2-polarised immune response (Grzych *et al.*, 1991; Pearce *et al.*, 1991). Indeed, *S. mansoni* soluble egg Ags (SEA) provide a potent stimulus

for Th2 responses *in vivo* (Okano *et al.*, 1999). Furthermore, following exposure to SEA, GMDCs, purified splenic DCs and human mo-DCs efficiently prime Th2 cells both *in vitro* and *in vivo* (de Jong *et al.*, 2002; Jankovic *et al.*, 2004; MacDonald *et al.*, 2001). Despite this, there is very little change in the surface phenotype of the DC populations examined in these studies following exposure to SEA (de Jong *et al.*, 2002; Jankovic *et al.*, 2004; MacDonald *et al.*, 2001). Nor do DCs respond to SEA exposure with high-level cytokine secretion (MacDonald *et al.*, 2001). Irrespective of this, DCs are both sufficient and necessary for Th2 induction against schistosome egg Ags, most clearly shown using CD11c depletion during murine infection (Jankovic *et al.*, 2004; MacDonald *et al.*, 2001; Phythian-Adams *et al.*, 2010). Studies using GMDCs have demonstrated the importance of CD40, MHC II and NFκB for the induction of SEA-specific Th2 responses (Artis *et al.*, 2005; MacDonald *et al.*, 2001; MacDonald *et al.*, 2002c), and are integral to our understanding of the mechanisms involved in DC priming of all Th2 responses, irrespective of their Ag-specificity. These studies have also shown that DC-derived IL-4 and IL-10 are not essential for this process (MacDonald and Pearce, 2002; Perona-Wright *et al.*, 2006a), whilst DC expression of OX40L is required to ensure sustained survival of SEA-specific Th2 responses (Jenkins *et al.*, 2007).

SEA is a complex mix of Ags, the majority of which are proteins, with over a thousand proteins identified (Mathieson and Wilson, 2010; Meevissen *et al.*, 2011). Many of these are glycoproteins (Meevissen *et al.*, 2011), which have been found to have immunostimulatory properties and bind to surface receptors on DCs. The glycosylation of these Ags seems integral to their ability to polarise Th2 responses, as, when SEA is pre-treated to remove glycans, Th2 activation is reduced (Faveeuw *et al.*, 2002; Okano *et al.*, 1999). Indeed, an SEA-derived glycan moiety, lacto-N-fucopentaose III (LNFPIII), binds to TLR4 on DCs, and can polarise Th2 responses (Okano *et al.*, 2001; Thomas *et al.*, 2003). Activation of distinct signalling pathways downstream of TLR4 may explain the ability of LNFPIII to induce Th2 responses following TLR4 ligation, despite the strongly Th1-polarised response elicited by the interaction of LPS with this receptor. TLR4 stimulation by LPS leads to the activation of three MAPK kinases (ERK, p38 and JNK) and prolonged NFκB stimulation, whilst LNFPIII activates ERK alone and NFκB only transiently (Thomas *et al.*, 2005). It has been postulated that these differences in signalling events may be orchestrated by the use of distinct co-receptors by the bacterial and helminth ligands.

Where LPS binds TLR4 in the presence of CD14, it may well be the CLR DC-SIGN that provides the necessary co-receptor in the case of LNFPIII (Hokke and Yazdanbakhsh, 2005).

Two glycoproteins, IPSEα-1 and omega-1, are major components of *S. mansoni* egg excretory/ secretory products (Dunne *et al.*, 1991; Schramm *et al.*, 2006). IPSEα-1 induces IgE production *in vivo*, which activates Ag-independent IL-4 production from basophils (Schramm *et al.*, 2007) – a potential early source of IL-4 during *S. mansoni* infection. Omega-1 is a T2 ribonuclease, whose uptake by DCs is facilitated by a CLR, the mannose receptor (MR) (Everts *et al.*, 2012; Everts *et al.*, 2009; Steinfelder *et al.*, 2009). Native purified or recombinant omega-1 is a potent inducer of Th2 responses, although omega-1 depleted SEA still retains Th2-polarising capacity (Everts *et al.*, 2009; Steinfelder *et al.*, 2009). Omega-1 appears to inhibit protein synthesis by degrading messenger and ribosomal RNA, and its ability to polarise DCs towards Th2 responses is dependent on its function as an RNase (Everts *et al.*, 2012). Omega-1 is not the only glycoprotein found in SEA that binds to a CLR on DCs, as other components of SEA have also been shown to bind to DC-SIGN and the macrophage galactose-type lectin, as well as MR (Meevissen *et al.*, 2011; van Liempt *et al.*, 2007). Following ligation of these receptors by SEA, the CLRs are rapidly internalised and targeted towards MHC II-containing endosomes, suggesting that these SEA ligands are presented to T cells (van Liempt *et al.*, 2007).

It is not only SEA-derived glycoproteins that play a role in polarising Th2 responses, as the schistosome-derived lipid phosphatidylserine (PS) polarises human mo-DCs towards Th2 induction, via TLR2, when cells are co-pulsed with LPS (van Riet *et al.*, 2009). However, another study has demonstrated that scishtosome-derived PS led to the generation of IL-10-producing T cells by human mo-DCs (van der Kleij *et al.*, 2002). It has also been proposed that dsRNA ligands present in SEA bind to TLR3 (Aksoy *et al.*, 2005), (discussed further in chapter 3). Despite the identification of a number of SEA-derived TLR-binding ligands, a number of studies have reported no requirement for MyD88, TLR2, TLR3 or TLR4 in the induction of schistosome-specific Th2 responses (Jankovic *et al.*, 2004; Kane *et al.*, 2004; Layland *et al.*, 2005; Vanhoutte *et al.*, 2008). Further to this, SEA and its components inhibit DC responses following TLR activation by bacterial ligands, such as LPS, or by whole bacteria, reducing the upregulation of MHC II and co-stimulatory

molecules, as well as IL-12 production, induced by these classical stimuli (Cervi *et al.*, 2004; Kane *et al.*, 2004; Perona-Wright *et al.*, 2012; Steinfelder *et al.*, 2009). This process may occur by the downmodulation of signalling pathways by SEA, particularly NFκB and MAPK (Kane *et al.*, 2004). However, SEA stimulates potentiated ERK signalling from human mo-DCs, which leads to the activation of c-Fos, a transcriptional repressor of IL-12 (Agrawal *et al.*, 2003). A recent study investigated whether SEA modulates the response of human mo-DCs to LPS by the inhibition of mTOR, a key metabolic pathway that integrates signalling responses to growth factors and nutrients, and is essential for DC growth and optimal function (Salmond and Zamoyska, 2011). However, whilst mTOR inhibitors skew DC responses towards Th2 and modulate LPS responses, neither omega-1 nor SEA appear to alter the activity of the mTOR pathway (Hussaarts *et al.*, 2013).

There are a number of open and interesting questions in the world of schistosome research that remain unanswered. These include a fuller understanding of the immune mediators involved in the protective response active in individuals who have acquired resistance to reinfection. There is also still much that is unknown about the role of DCs in the orchestration of immune responses during schistosome infection. Over the past ten or so years, our understanding of the processes involved in DC-mediated immune activation against schistosomes has improved significantly (Perona-Wright *et al.*, 2006b). However, there are a number of unknowns surrounding DC interactions with schistosome Ags, and how these shape consequent immune activation. Whilst we know that DCs are essential for optimal Th2 induction against *S. mansoni* (Phythian-Adams *et al.*, 2010), it is not yet clear which DC subset(s) are involved in orchestrating this response, or indeed, whether any DC subsets found *in vivo* inhibit or regulate the developing Th2 response. A number of important schistosome egg Ags have been identified that stimulate Th2 polarisation (Everts *et al.*, 2009; Schramm *et al.*, 2006; Steinfelder *et al.*, 2009), however there are likely to be other egg Ags that interact with PRRs on DCs that are yet to be identified. There is also still further work to be done to characterise the intracellular responses of DCs following exposure to schistosome Ags – what are the gene expression changes and which signalling pathways are modulated following the binding of schistosome Ags to PRRs? How do these changes affect DC phenotype and function? Furthermore, how do schistosome egg Ags modulate DC responses to bacteria, above and beyond the modulation of protein synthesis by omega-1? How does inhibition of protein synthesis by

omega-1 skew DCs towards Th2 polarisation? Despite continuing work in the area, a bone fide DC-derived signal 3 that is integral to Th2 polarisation has yet to be identified. Although IFN-I have been shown to play a role in a variety of settings, in immune responses against a range of pathogens, the role of this pluripotent cytokine family in Th2 responses has not been investigated. Thus, there are still lots of exciting areas of research to explore before our understanding of DC-schistosome interactions is complete.

1.6.3 *Heligomosoides polygyrus* infection

H. polygyrus is an intestinal nematode parasite, common in wild mouse populations, and widely used as a laboratory model of intestinal helminth infection. It is related to human hookworms and nematodes that afflict livestock (Maizels *et al.*, 2012b). The third larval stage is infective and larvae enter the host orally, invading the small intestinal wall within 24h and remaining in the submucosa for ~8d, undergoing two molts in that time (Bryant, 1973; Reynolds *et al.*, 2012). Adult worms emerge and feed on intestinal tissue (Bansemir and Sukhdeo, 1994), coiling around the villi of the small intestinal epithelium. At this stage worms mate and egg production begins. Although *H. polygyrus* worms start to be expelled from the host around 4 weeks after infection, they can persist within the host for many months, resulting in chronic infection (Monroy and Enriquez, 1992). Persistence is measured by determining the number of eggs released in the faeces and counting the worms that remain in the small intestine (Reynolds *et al.*, 2012).

Impaired worm expulsion in severe combined immunodeficient (SCID) mice demonstrates that the adaptive immune system has an important role in orchestrating this response (Hashimoto *et al.*, 2009). Anti-CD4 treatment results in an increase in worm fecundity (Urban *et al.*, 1991a), whilst transfer of T effectors ($CD4^+$ $CD25^-$) from the MLN of chronically infected mice leads to lower worm burden in recipient animals (Hashimoto *et al.*, 2009). These studies demonstrate the importance of $CD4^+$ T cells in controlling expulsion of this intestinal nematode. $CD4^+$ T cell production of IL-4 is the primary mediator that promotes worm expulsion and limits egg production during *H. polygyrus* infection (Svetic *et al.*, 1993; Urban *et al.*, 1991b). The exact mechanisms of worm expulsion are not known, but likely involve both innate effectors and non-immune cells (Reynolds *et al.*, 2012). Processes that may be involved in *H. polygyrus* expulsion include the ‘weep and sweep’ response, which is characterised by an increase in luminal fluid and

smooth muscle contractility in the intestine (Anthony *et al.*, 2007). Mast cells are also thought to be major players in *H. polygyrus* expulsion (Behnke *et al.*, 2003).

$CD4^+$ $Foxp3^+$ Treg numbers increase dramatically in the MLN during infection with *H. polygyrus* (Finney *et al.*, 2007; Rausch *et al.*, 2008). Treg induction likely benefits both the parasite and host. A TGFβ-like molecule secreted by *H. polygyrus* drives Treg differentiation, and inhibition of TGFβ during infection causes a reduction in worm burden (Grainger *et al.*, 2010), suggesting that induction of Tregs aids worm persistence. Depletion of Tregs early in infection also leads to enhanced Th2 responses and increased intestinal pathology, intimating that Tregs also provide protection to the host (Rausch *et al.*, 2009).

Similarly to *S. mansoni* infection, depletion of $CD11c^+$ cells during *H. polygyrus* infection inhibits the induction of the Th2 response in the MLN (Smith *et al.*, 2011), highlighting the essential role of DCs in activating the adaptive immune response against this intestinal helminth. Studies have shown that CD80 and CD86 signalling are essential to optimal IL-4 induction and the Th2 response (Greenwald *et al.*, 1997; Lu *et al.*, 1994), whilst OX40L is also required to promote IL-4 production from T cells during *H. polygyrus* infection (Ekkens *et al.*, 2003).

1.7 Immune recognition of bacteria

Heat-killed preparations of the Th1/Th17-polarising gram-negative bacteria *Salmonella typhimurium* (St) and the gram-positive bacteria *Propionibacterium acnes* (Pa) are used in this thesis as a counterpoint to the Th2-polarising SEA. St is an anaerobic intracellular pathogen, which, when live and unattenuated, causes severe diarrhoeal disease (Garai *et al.*, 2012). Recognition of St is mediated entirely via TLRs, as BMMΦs lacking both MyD88 and TRIF do not respond to heat-killed St (Arpaia *et al.*, 2011). TLR2, TLR4 and TLR5 mediate the majority of St recognition and an additional role for TLR9 was confirmed using TLR2, TLR4 and TLR9 double and triple knock out mouse strains (Arpaia *et al.*, 2011). St flagellin is recognised by TLR5 (Feuillet *et al.*, 2006). Recognition of St by the intracellular TLRs, TLR7 or TLR3 has also been suggested, as the use of an inhibitor of endosome acidification blocked the remaining TNFα induced by St in TLR2-TLR4-TLR9 combined knockout BMMΦs (Arpaia *et al.*, 2011).

Pa is the most abundant bacterial species in acne lesions (Jugeau *et al.*, 2005). Pa-sensing is dependent on TLR2, with no requirement for TLR1, TLR4 or TLR6 for the induction of Pa-specific IL-12 from human monocytes (Jugeau *et al.*, 2005; Kim *et al.*, 2002). St and Pa potently activate GMDCs, inducing upregulation of MHC II and co-stimulatory molecules, and stimulating the secretion of a range of pro-inflammatory cytokines, including IL-12, IL-6 and TNFα (Perona-Wright *et al.*, 2009; Perona-Wright *et al.*, 2012). Both St and Pa-experienced GMDCs efficiently induce strain-specific IFNγ and IL-17 responses *in vivo* (Perona-Wright *et al.*, 2009; Perona-Wright *et al.*, 2012).

1.8 Thesis aims

Broadly, the work detailed in this thesis aimed to dissect the role of different DC subsets in the induction of Th2 responses and identify the importance of IFN-I in Th2 immunity.

Key questions:

How do cDC and pDC subsets respond to stimulation with the potently Th2-polarising soluble egg Ags (SEA) from *S. mansoni*?

- Does exposure to SEA alter the surface phenotype of murine cDCs and pDCs generated *in vitro* using Flt3-L?
- Do FL-cDCs and pDCs produce SEA-specific cytokines?
- Is the phenotype displayed by FL-cDCs following exposure to SEA distinct from GMDCs?

Can Flt3-L-dependent DCs polarise SEA-specific Th2 responses?

- Do FLDC subsets migrate efficiently to the dLN?
- On what factors do FLDCs depend for Th2 polarisation?
- Is IFN-I signalling required for FLDC Th2 induction?

Is IFN-I active during Th2 responses *in vivo*?

- Do splenic cDCs and human blood-derived DCs display a similar phenotype to FLDCs following exposure to SEA?
- Is systemic IFN-I detectable in murine and human *S. mansoni* infection?
- Is IFN-I signalling involved in the development of the immune response during murine models of helminth infection?

How are schistosome-specific Th2 responses orchestrated in the intestine?

- How are intestinal DC subsets affected by *S. mansoni* infection?
- Are intestinal DCs involved in the induction of the egg-specific response at this site?

Table 1.1 TLRs and their ligands

Receptor	Dimerises with	Example ligands	Origin of ligand	Adaptor protein	Localisation
TLR2	TLR1 TLR6	Triacylated lipopeptides Diacylated lipopeptides	Bacteria, viruses, parasites, self	MyD88, MAL	Plasma membrane
TLR3	TLR3	dsRNA	Viruses, parasites?	TRIF	Endolysosome
TLR4	TLR4	LPS	Bacteria	MyD88, TRIF, MAL, TRAM, SARM	Plasma membrane, endolysosome
TLR5	TLR5	Flagellin	Bacteria	MyD88	Plasma membrane
TLR7/ TLR8	TLR7/ TLR8	ssRNA	Bacteria, viruses, self	MyD88	Endolysosome
TLR9	TLR9	CpG DNA	Bacteria, viruses, protozoa, self	MyD88	Endolysosome

Table 1.2 TLR expression by mouse DC subsets

	In vivo (splenic) & *in vitro* FLDCs (Flt3-L)			*In vitro* BMDCs
Receptor	**CD8α$^+$ cDCs**	**CD11b$^+$ cDCs**	**pDCs**	**GM-CSF**
TLR1	++	++	+	+
TLR2	++	++	+	+
TLR3	++	-	-	-
TLR4	+	++	-	++
TLR5	-	++	+	+
TLR6	++	++	+	+
TLR7	-	++	++	+
TLR9	+	+	++	+

Table 1.3 TLR expression by human DC subsets

	PBMC subsets			*In vitro* mo-DCs
Receptor	**CD141$^+$ cDCs**	**CD1c$^+$ cDCs**	**pDCs**	**GM-CSF + IL-4**
TLR1	++	++	++	+
TLR2	+	++	-	++
TLR3	++	-	-	+
TLR4	-	++	-	++
TLR5	-	++	-	+
TLR6	++	+	++	++
TLR7	-	+	++	-
TLR8	+	++	+	+
TLR9	-	-	++	+

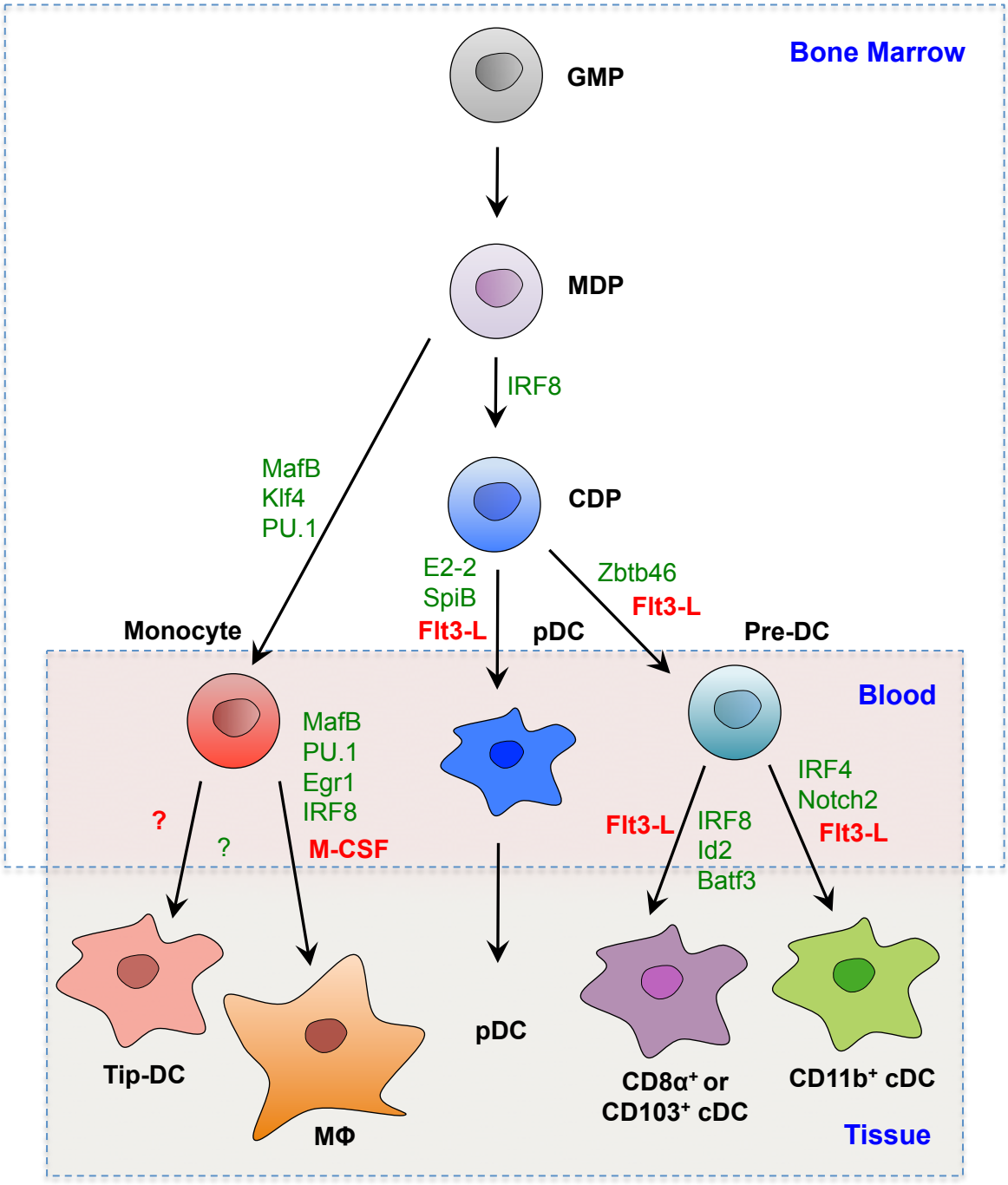


Figure 1.1 Myeloid cell development
TFs involved in development are included in green, growth factors in red. The early developmental events occur in the BM. The MΦ-DC progenitor (MDP) gives rise to monocytes and the common-DC precursor (CDP). CDPs can differentiate into pDCs or the cDC precursor, pre-DCs. pDCs are fully differentiated in the BM and migrate to the periphery via the blood. Pre-DCs migrate via the blood to the periphery, where they can differentiate into the cDC subsets. Monocytes are also found circulating in the blood, giving rise to MΦs in the periphery. Under inflammatory conditions, monocytes can also give rise to a population of DC-like cells that produce high levels of TNFα and iNOS (Tip-DCs). GMP = Granulocyte-MΦ progenitor. Figure is adapted from Satpathy *et al.* (2012).

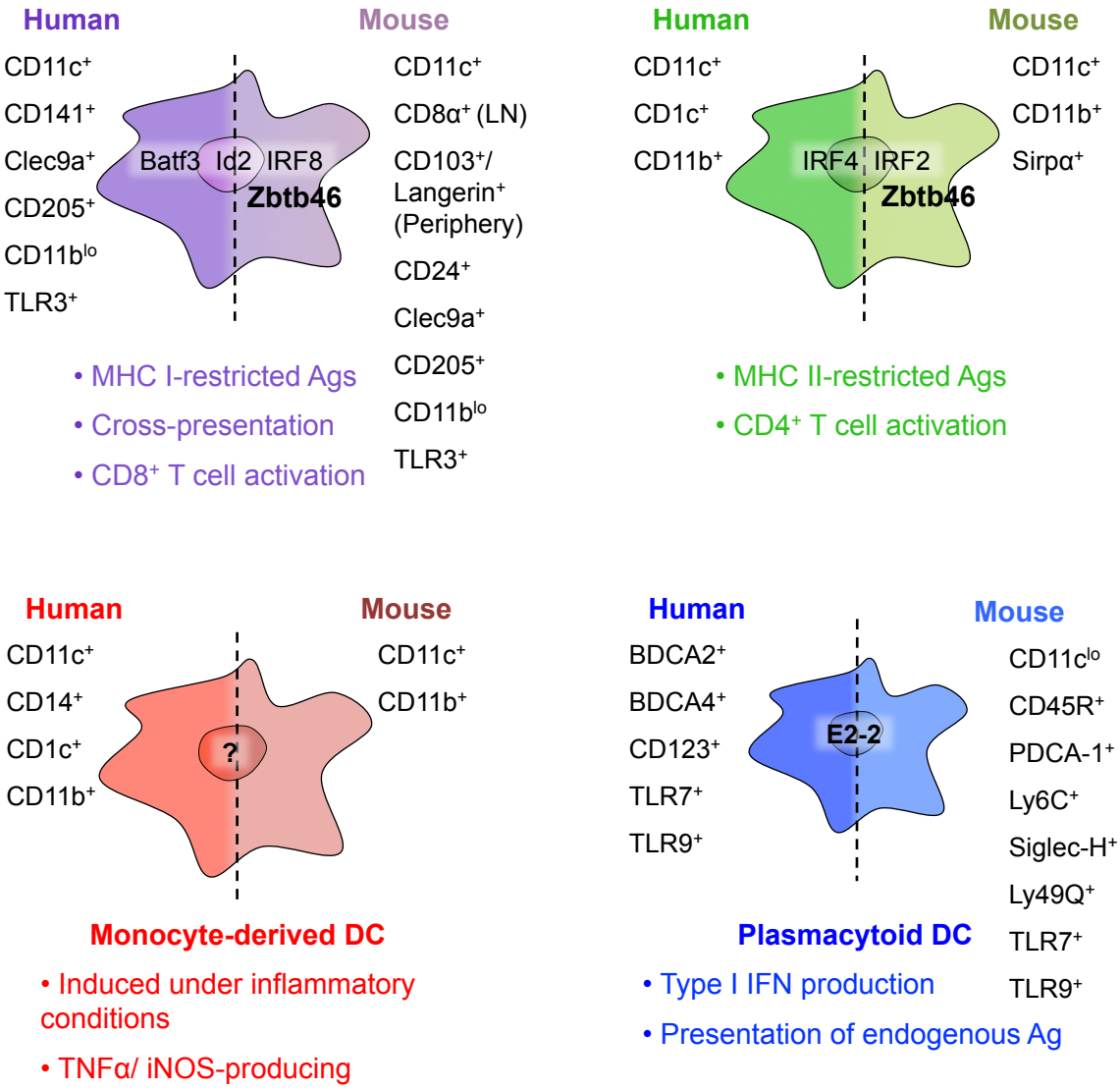


Figure 1.2 DC subsets in human and mouse
This figure depicts the surface markers associated with the different DC subsets in mouse and human, and their characteristics and functions. The TFs associated with each subset are also shown, including the cDC TF, Zbtb46. The pDC TF, E2-2, has been shown to be active in human pDC homeostasis (Ghosh *et al*., 2010). Batf3 knockdown studies in human CD141+ DCs suggests that this TF is active in this subset (Poulin *et al*., 2012). It is not yet known whether the other TFs are involved in human DC development. Adapted from Collin *et al.* (2011).

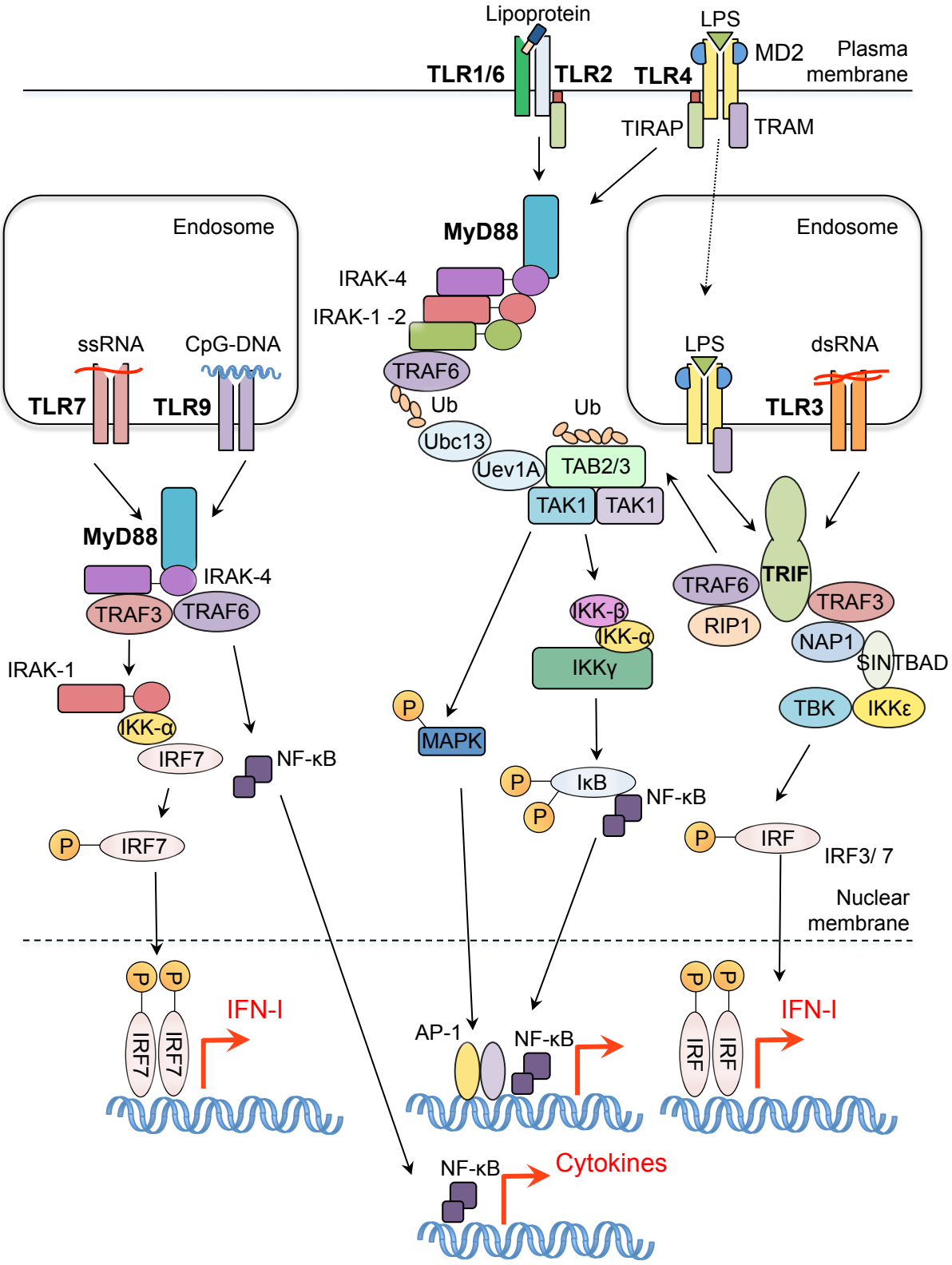


Figure 1.3 TLR signalling pathways
This figure includes the signalling pathways downstream of the main TLRs. Adapted from Takeuchi and Akira (2010).

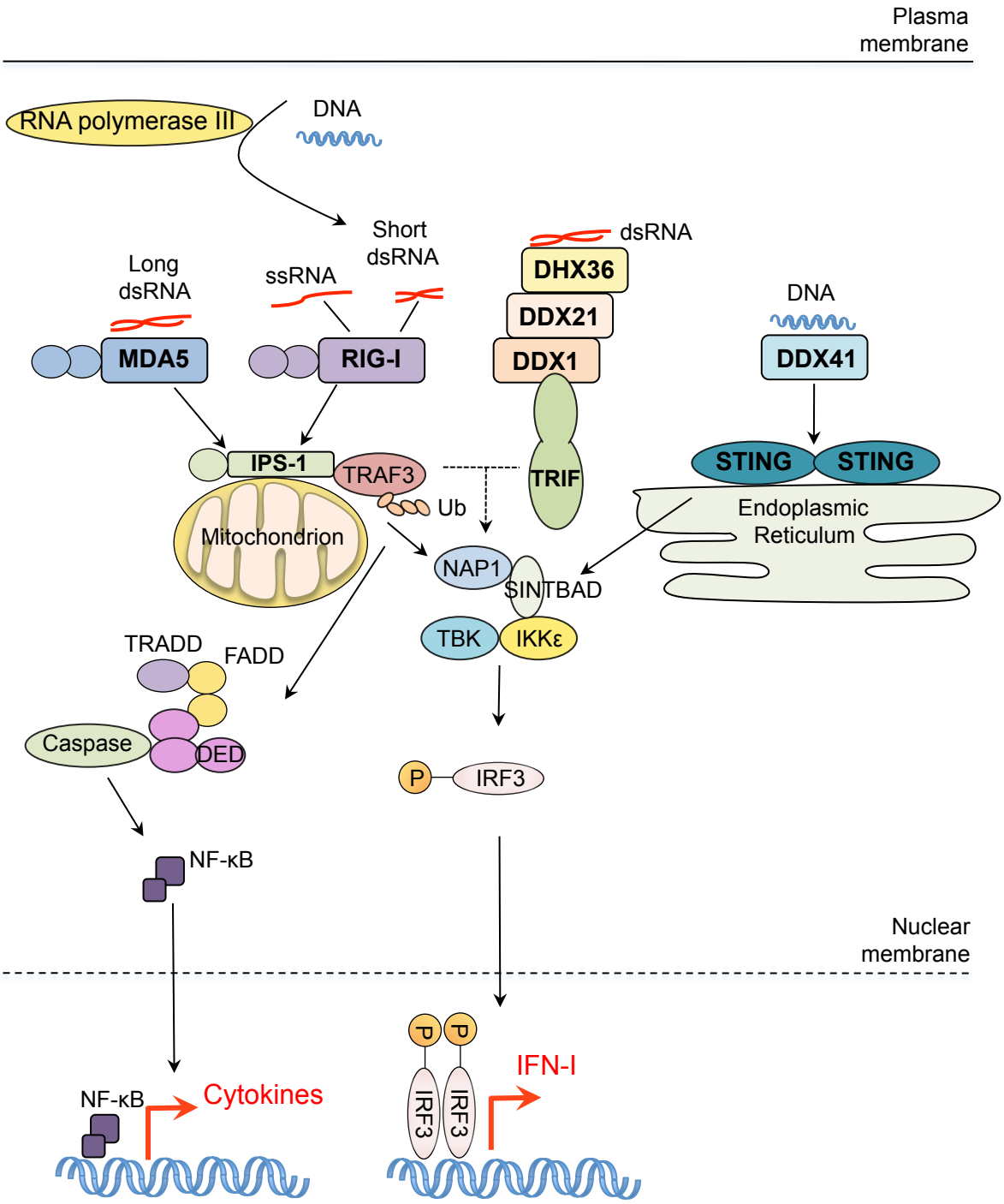


Figure 1.4 Signalling pathways of the cytosolic helicase nucleic acid sensors involved in the IFN-I response

All of these nucleic acid sensors share a common helicase domain but use a range of different adaptor proteins. The RLRs, MDA5 and RIG-I signal via IPS-1, which is associated with the mitochondrial membrane. The RLRs recognise RNA ligands. RNA polymerase III can convert DNA, including self DNA, into dsRNA ligands that can be recognised by RIG-I. DDX41 is one of a growing family of cytosolic sensors that utilises the adaptor protein STING. DDX1-DDX21-DHX36 co-operate to recognise dsRNA and induce IFN-I production downstream of TRIF. Figure is adapted from Takeuchi and Akira (2010) and Broz and Monack (2013), including data from Zhang *et al*. (2011).

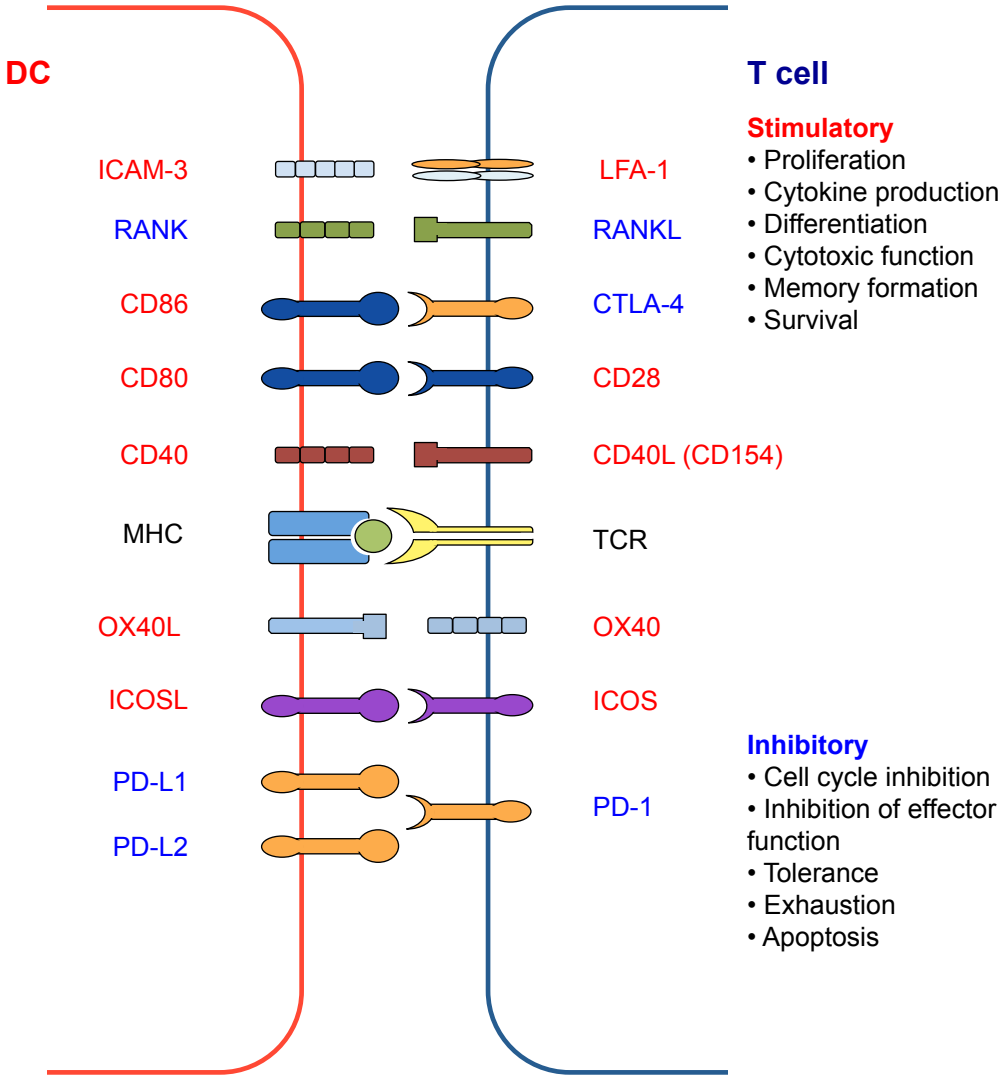


Figure 1.5 DC-T cell interactions
The ligands and receptors expressed by DCs and T cells that facilitate their interaction and form part of the immunological synapse. Co-stimulatory molecules are depicted in red, co-inhibitory molecules in blue. Stimulatory and inhibitory signals impact on the turnover and function of T cells. Adapted from Chen and Flies (2013)

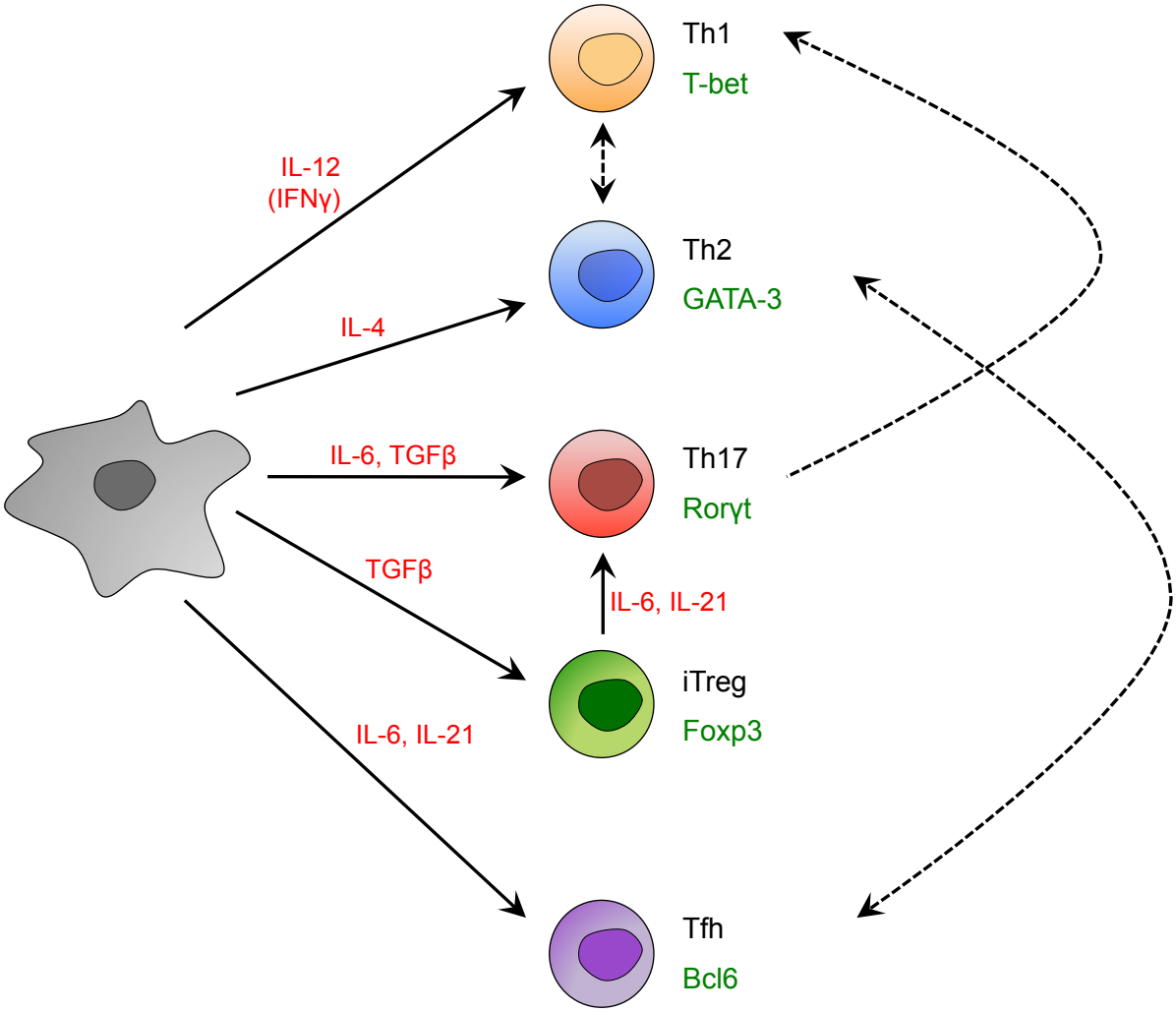
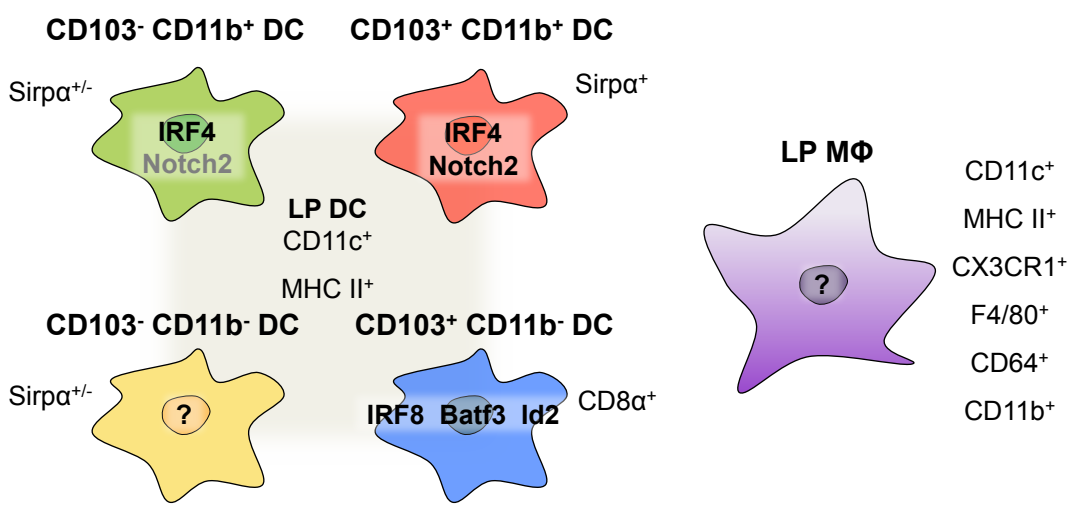


Figure 1.6 T cell subsets and T cell plasticity
Highlighted in red are the cytokines involved in the differentiation of the different T cell subsets. IFNγ can enhance the polarisation of Th1 cells, but is not produced by DCs. The TFs associated with each T cell subset are depicted in green. There is some plasticity in T cell fate, with reports that Th1 and Th2 cells can be converted to the alternative subset. Under certain conditions, iTregs can be converted to Th17 cells, and Th17 cells can display characteristics of the Th1 lineage. It has also been shown that Tfh cells produce Th2 cytokines in the reactive LN during helminth infection. Adapted from Zhou *et al.* (2009)

A.



B.

	CD103⁻ CD11b⁺ LP DCs	CD103⁺ CD11b⁺ LP DCs	CD103⁻ CD11b⁻ LP DCs	CD103⁺ CD11b⁻ LP DCs	LP MΦs
Precursor	Pre-DC	Pre-DC	Pre-DC	Pre-DC	Monocyte
Differentiation factor	Flt3-L	Flt3-L	Flt3-L	Flt3-L	M-CSF
Homeostatic factor	GM-CSF	GM-CSF	GM-CSF	GM-CSF	?
Localisation in LP	?	Villus core & PPs/ ILFs	?	Prevalent in PPs/ ILFs	Close to epithelium
T cell priming	+	++	+	+	-
Migration to MLN	+	++	+	+	-
Gut imprinting	+	+	+	++	-
RALDH activity	+	+	+	+	-
Function	• Th17 polarisation during bacterial infection?	• Treg induction in steady-state • Th17 polarisation during bacterial infection	?	Cross-presentation?	• Ag sampling & destruction • Treg activation • Maintenance of tolerance

Figure 1.7 Intestinal DCs and MΦs
LP DC subsets and MΦ surface markers and TFs (bold) (A). The characteristics of LP DC subsets and MΦs. ? signifies no data currently available. Data collated from Bogunovic *et al*. (2009), Cerovic *et al*. (2012), Coombes *et al*. (2007), Ginhoux *et al*. (2009), Greter *et al*. (2012), Hadis *et al*. (2011), Johansson-Lindbom *et al*. (2005), Persson *et al*. (2013), Satpathy *et al*. (2013), Schulz *et al*. (2009), Varol *et al*. (2010).

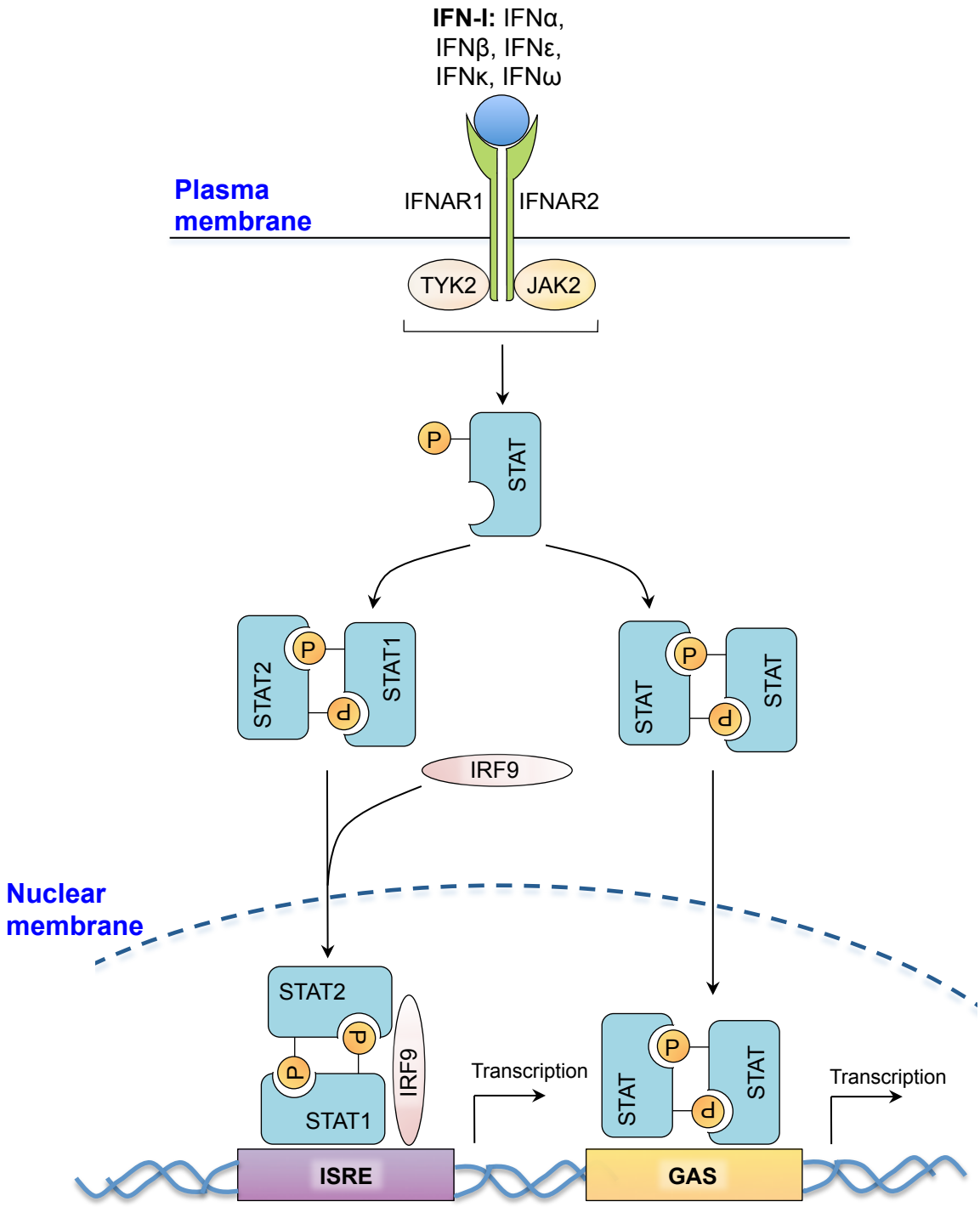


Figure 1.8 IFNAR activation of JAK-STAT dependent signalling
Binding of IFN-I leads to reorganisation and heterodimerisation of the receptor subunits, IFNAR1 and IFNAR2, leading to the autophosphorylation of the receptor-associated JAKs, TYK2 and JAK2. The activated kinases then phosphorylate STATs. The formation of a STAT1-STAT2 heterodimer, with the addition of IRF9, leads to the formation of a complex called IFN-stimulated gene factor 3 (ISGF3). This complex can bind to a region upstream of IFN-stimulated genes (ISGs) called the IFN-stimulated response element (ISRE). STAT complexes that do not recruit IRF9 (primarily STAT1, STAT2, STAT3 and STAT5) can bind to IFNγ-activated sites (GAS) found in the promoter regions of ISGs. Some genes have both ISRE and GAS elements in their promoter region, whilst others only have one. Thus, different STATs, and consequently different IFN-I subtypes, are required for their activation. Adapted from Platanias *et al*. (2005).

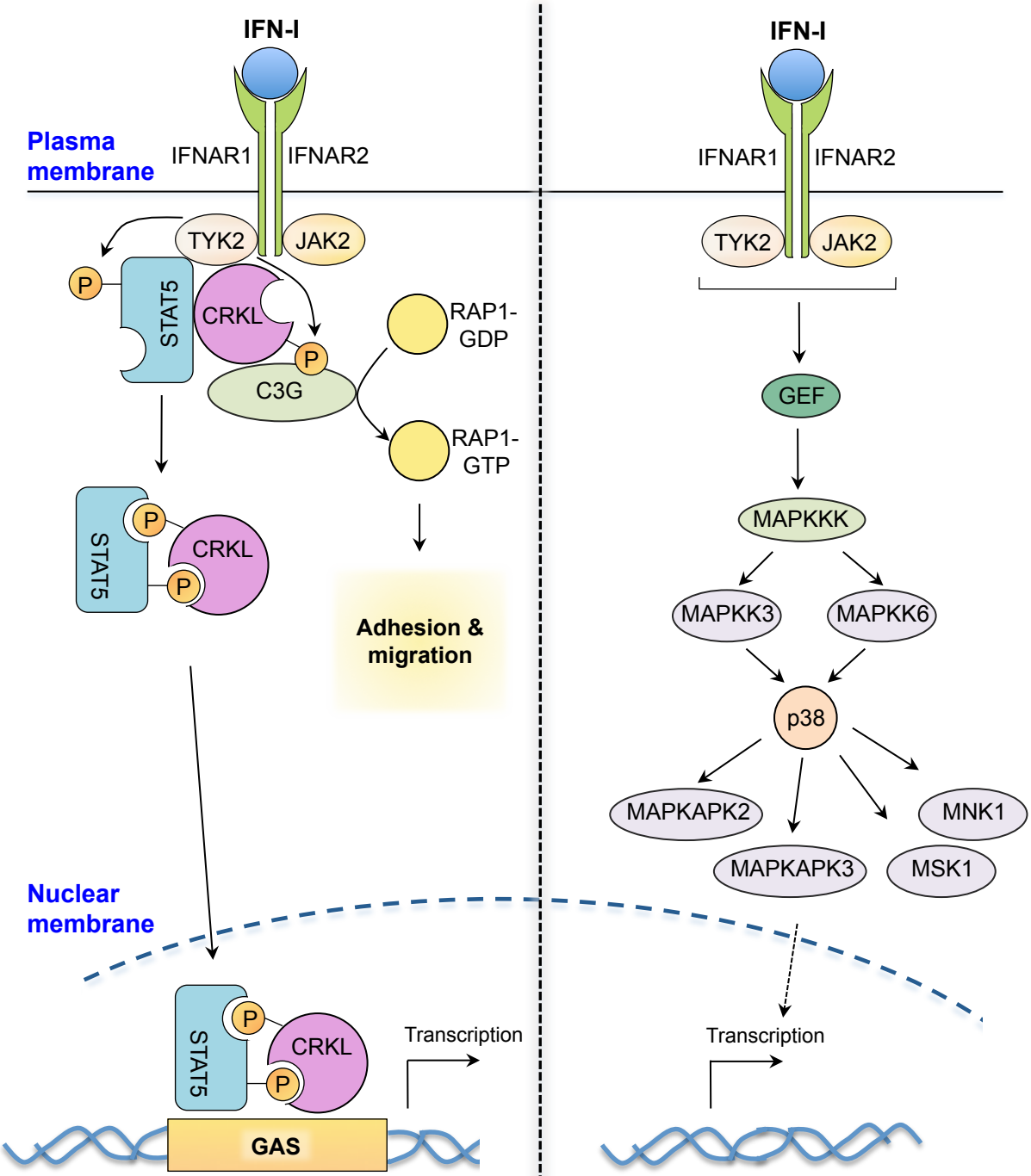


Figure 1.9a CRKL-mediated signalling downstream of IFNAR
Following activation of TYK2, STAT5 and CRK-like (CRKL) are phosphorylated. These proteins form a complex that translocates to the nucleus and can bind certain GAS elements upstream of certain ISGs. Activation of CRKL also facilitates the guanine-nucleotide-exchange (GEF) activity of C3G, leading to the conversion of RAP1-GDP to RAP1-GTP. This activates the GTPase, allowing it to interact with and activate effector molecules involved in cell adhesion and migration.

Figure 1.9b Activation of MAPK p38 downstream of IFNAR
IFNAR-activated JAKs regulate the phosphorylation of GEFs, which leads to the activation of a MAPKKK, leading to the downstream activation of MAPKK3 & MAPKK6, which directly phosphorylate p38, resulting in its activation. Activated p38 regulates activation of multiple effectors, including MAPK-activated protein kinase 2 (MAPKAPK2), MAPKAPK3, mitogen- & stress-activated kinase 1 (MSK1) & MAPK-interacting protein kinase 1(MNK1).

Chapter 2. MATERIALS AND METHODS

2.1 Mice, infections and *in vivo* experiments

2.1.1 Mice

C57BL/6, CBA, BALB/c, *Ab*$^{-/-}$ (C57BL/6)(Grusby *et al.*, 1991), B-Act dsRed (C57BL/6)(Vintersten *et al.*, 2004), *Cd40*$^{-/-}$ (C57BL/6)(Ensminger *et al.*, 2001), CD45.1 (C57BL/6), CX3CR1eGFP (C57BL/6)(Jung *et al.*, 2000), CD11c.DOG (C57BL/6)(Hochweller *et al.*, 2008), *Ifnar1*$^{-/-}$ (C57BL/6)(Hwang *et al.*, 1995), *Il10*$^{-/-}$ (C57BL/6)(Kuhn *et al.*, 1993), IL-10eGFP (Kamanaka *et al.*, 2006) x KN2 (Mohrs *et al.*, 2005)(C57BL/6), IL-13eGFP (Neill *et al.*, 2010) x KN2 (C57BL/6), KN2 (C57BL/6), Ly5.1xOTII (C57BL/6)(Barnden *et al.*, 1998), *Myd88*$^{-/-}$/*Trif*$^{-/-}$ (C57BL/6), *Myd88*$^{-/-}$ (C57BL/6)(Adachi *et al.*, 1998), *Trif*$^{-/-}$ (C57BL/6)(Yamamoto *et al.*, 2003), *Tlr9*$^{-/-}$ (C57BL/6)(Hemmi *et al.*, 2000) mice were maintained under specific-pathogen free conditions at the University of Edinburgh Animal Facilities. *Ly75*$^{-/-}$ (C57BL/6)(Guo *et al.*, 2000) mice were maintained under specific-pathogen free conditions at the Burnet Institute Animal Facilities, Melbourne, Australia. *Ifnar1*$^{-/-}$ and littermate control bone marrow was originally a generous donation from Dr. Caetano Reis e Sousa, London Research Institute, CRUK. Dr Reis e Sousa also generously provided mice to set up an *Ifnar1*$^{-/-}$ mouse colony at UoE. All experiments were approved under a Project License granted by the Home Office (U.K.) and conducted in accordance with local guidelines.

2.1.2 Subcutaneous injections into feet

BMDC transfer: WT or knockout BMDCs were cultured as below (section 2.3 and 2.4), with SEA, St or in medium alone. 5 x 10^5 BMDCs were injected subcutaneously into the feet of recipient WT C57BL/6 mice (2.5 x 10^5 per foot), as previously described (Cook *et al.*, 2012). 7 days following transfer the draining popliteal LNs were harvested. Single cell suspensions of LN cells (1 x 10^6 cell/ml) were cultured in X-vivo 15 medium (Lonza) containing 2mM L-Glutamine and 50 mM 2-ME (Invitrogen) in 96 well plates at 37°C in a humidified atmosphere of 5% CO_2 with or without 15µg/ml SEA, or 1µg/ml St. Supernatants were harvested from the cultures after 72h and cytokine production assessed by ELISA.

BMDC tracking: dsRed or CD45.2 BMDCs were cultured as below (section 2.3 and 2.4), with SEA, St or in medium alone. 2 x 10^6 BMDCs were injected subcutaneously into the feet of recipient WT C57BL/6 or CD45.1 mice (1 x 10^6 per foot), this DC dose was based on unpublished optimization experiments performed by lab members (Rachel Lundie, Alex Phythian-Adams), that allowed optimal visualisation of transferred cells within the first 72h following injection. 24, 48 or 72h following transfer the draining popliteal LNs were harvested. 2 pLNs from each group were collected and prepared for microscopy, to prevent loss of the dsRed signal, pLNs were prepared as described in section 2.7.4.

For flow analysis, pLNs were digested at 37°C for 30 min with 1.75 Wunsch Units/ml Liberase TI (Roche Diagnostics) and 80 Kunitz Units/ml DNase I type VI (Sigma) in HBSS (Sigma) containing 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen). 100µl 0.1M pH 7.3 EDTA (Gibco) stop solution per ml was then added and the tube topped up with DMEM containing 50U/ml penicillin and 50µg/ml streptomycin. The resulting suspension was then passed through a 70µm cell strainer to obtain a single cell suspension, and cells counted and resuspended for use. Cells were then stained for analysis by flow cytometry.

For egg injections: The mice were immunised s.c. in the top of each rear foot with 2,500 eggs in 50µl PBS or with 50µl of PBS alone, as described (Phythian-Adams *et al.*, 2010).

2.1.3 SEA intravenous injections

Mice were injected intravenously with 50µg of SEA in 200µl PBS or with 200µl PBS alone. Spleens were harvested 12h later and diced, before being digested as above (section 2.1.2, BMDC tracking) for 15 min. This dose was chosen to approximate the dose of SEA used to stimulate BMDCs *in vitro* (25µg/ml). The blood volume of a mouse is estimated to be 77-80µl/g (Jackson Laboratory), which for a 20-25g mouse is 1.5-2ml, thus 50µg equates to roughly 25µg/ml. Optimisation experiments using 50 or 100µg of SEA demonstrated that the lower dose was sufficient to elicit a phenotype from splenic DCs within 12h of administration. An intravenous route of delivery was chosen to maximize the amount of Ag that would reach the spleen.

Low-density cells were enriched using NycoDenz (1.077g.cm3; Axis-Shield) density gradient separation and non-DC lineage cells were coated with biotinylated mAbs against

murine CD2, CD3ε, CD49b, mIgM and erythrocytes (Ter-119), and depleted using MyOne Streptavidin Dynabeads (Dynabeads Mouse DC Enrichment Kit; Invitrogen). This purification protocol was optimised in the MacDonald Laboratory (Rachel Lundie *et al*., manuscript submitted). $CD11c^{hi}$ $B220^{-}$ cDCs were then sorted from $B220^{+}$ $CD11c^{mid}$ pDCs using a BD FACs Aria II. Approximately 100,000 cDCs were sorted directly into 1ml aliquots of Trizol (Invitrogen) and snap frozen on dry ice.

2.1.4 *S. mansoni* infection

Cercariae were shed from patent *S. mansoni* infected *B. glabrata* snails by placing the snails in pond water under a heat lamp for 50 minutes. A sample of cercariae was counted in a 1:1 dilution of Lugol's iodine solution under a dissection microscope. Female C57BL/6 mice were anaesthetised with 0.01ml/g of 0.25ml Domitor (Janssen) and 0.19ml Vetalar (Pfizer) mixed in 2.6ml PBS (Sigma). For MLNX experiments, male mice were anaesthetized with 0.1ml/g of 0.5ml Vetalar and 0.25ml Rompun (Bayer) mixed in 4.25ml H_20 (Sigma). Fur was removed from abdomen with clippers, and animals taped to a pre-warmed heat pad to help maintain body temperature during the procedure. A stainless steel ring was taped over the shaved part of the abdomen, 40-180 cercariae were placed into the ring in 200µl of pond water, with an additional 200µl of pond water added on top. Cercariae were allowed to penetrate for 30 minutes, following this time, steel ring and remaining cercariae were removed from the animals, and animals were revived by a subcutaneous injection of 0.1ml of 0.04ml antisedan diluted in 0.96ml PBS (Sigma). Following infection animals were monitored with weights measured 3 times a week starting at week 5. On d49 or 56 organs were harvested following CO_2 asphyxiation.

2.1.5 Serum collection

Blood was collected non-terminally via puncture of the submandibular vein using a 26-gauge needle. When blood was collected at experimental endpoints, mice were first anaesthetised by a 100µl subcutaneous injection containing 50µl 100 mg/ml Vetlar (Pfizer) and 50µl 1 mg/ml Domitor (Janssen), and blood was taken from the brachial artery before mice were culled by a Schedule 1 method. Blood was collected into Microtainer tubes (BD), which were spun at 13,000 rpm at 4 °C for 10 minutes. Serum was removed from the pellets and frozen at -80 °C until use.

2.1.6 *H. polygyrus* infection

Mice were infected with 200 *H. polygyrus* L3s in 200µl of dH_2O by oral gavage, using a 24-gauge feeding needle, as previously described (Hewitson *et al.*, 2011). Organs were harvested on d7 of infection following cervical dislocation.

2.1.7 SEA/ *S. mansoni* egg gavage

Mice were gavaged with 10-100µg SEA, 2500 *S. mansoni* eggs in PBS or PBS alone. MLNs were harvested 7d later and single cell suspensions prepared and restimulated with 15µg/ml SEA, 16.65µg/ml αCD3 (prepared in house) or in medium alone, as described in section 2.1.2, BMDC transfer. Supernatants were harvested from the cultures after 72h and cytokine production assessed by ELISA.

2.1.8 Subserosal injection of *S. mansoni* eggs

Prior to surgery, mice received analgesia, Rimadyl (Pfizer) 1:100 dilution of stock, 0.075ml per 10g body weight and Vetergesic 1:10 dilution of stock, 0.015ml per 10g body weight. Mice were placed under general anaesthetic using Isoflurane in oxygen and abdomen fur removed with clippers. Skin was sterilised using 70% ethanol, a short (~1cm) midline incision was made in the skin and then in the peritoneal wall. The intestine was then removed from the abdomen using cotton buds and placed on gauze soaked in saline. To inject, the intestine was gently gripped with tweezers and an insulin needle used to pierce the outer lining of the intestine. 1000 *S. mansoni* eggs in PBS or PBS alone were injected into the subserosa. This dose of eggs was chosen as an approximation of the average daily egg dose that a mouse might be exposed to, with ~4-5 fecund worm pairs producing ~300 eggs per day. The intestine was returned to the abdomen, and the peritoneal wall closed by suturing. The skin was sealed using surgical staples. Simon Milling and members of his laboratory at the University of Glasgow developed this experimental protocol. In CD11c depletion experiments, mice were injected intraperitoneally with 16ng/g of DTx dissolved in H_20 daily, from 2d prior to surgery and 6d after. This dose was adapted from Phythian-Adams *et al*. (2010), where 8ng/g of DTx was administered daily. A 16ng/g dose was found to be safe and more effective than 8ng/g (Alex Phythian-Adams, unpublished findings). Organs were harvest 7d after subserosal egg injection and prepared as described in section 2.2.1.

2.1.9 Mesenteric lymphadenectomy

Mice received analgesia prior to surgery and were anaesthetised as above (section 2.1.8). The protocol is very similar to that described for rats by Milling *et al*. 2006 and was further adapted for use in mice by Simon Milling's laboratory. The intestine was exposed as in section 2.1.8 and the MLN carefully excised using tweezers, mice were then closed up as above. After 10d, surgical staples were removed. 28d after surgery mice were infected with *S. mansoni* cercariae as described in section 2.1.4.

2.2 Parasitology and pathology of helminth infected mice

2.2.1 *S. mansoni* worm counts

Following CO_2 asphyxiation, the hepatic portal vein was cut and mice perfused by injecting saline solution into the heart. This perfusate was collected from infected animals in order to enumerate the number of worms flushed out of the mesenteric veins. Samples were spun at 400xg for 15 min and excess perfusate removed with a stripette. The remaining blood/ worm mixture was diluted in 3 ml of 1x FACS fix/ lysis solution (BD). After incubating for 2 min, this mixture was rinsed through a 40µm cell strainer using PBS. The sieve was then inverted on a petri dish and rinsed again with PBS to remove any worms. Female and male worms could then be identified using a dissection microscope. Females were identified as longer thinner and often darkened by haemozoin. Males were fatter, shorter and much lighter in colour than females.

2.2.2 *S. mansoni* egg counts

To determine the number of parasite eggs present in the liver and intestine, tissue samples were weighed before being digested overnight at 37°C using 4% KOH dissolved in H_2O. The numbers of eggs present in 100µl of digested tissue could then be determined using a microscope. For each sample, the average count from 3x 100µl samples was calculated. The number of eggs per gram of tissue could then be extrapolated, taking into account the weight of the tissue sample and the total tissue weight when harvested.

2.2.3 Hepatomegaly and splenomegaly of *S. mansoni* infected mice

After CO_2 asphyxiation, the mouse weight, and liver and spleen weights were recorded. Hepatomegaly and splenomegaly could then be calculated, defined as spleen and liver size as a proportion of total body weight of naïve and infected animals.

2.2.4 Egg and worm counts from *H. polygyrus* infected mice

Faecal egg counts: Faeces were weighed and left to form a homogenous solution in 2ml of distilled H_2O. An equal volume of saturated NaCl was added, and the resulting solution loaded to fill one chamber of an egg counting slide (McMaster 2 cell counter; Hawksley). Eggs within the grid were enumerated using a light microscope, and eggs per gram of faeces were calculated using the following calculation:

$$\text{Eggs/ g of faeces: } \frac{\text{egg count x 26.67*}}{\text{faeces mass (g)}}$$

$$\text{*calculated as: } \frac{\text{volume of liquid faeces dissolved in (4 ml)}}{\text{chamber volume (0.15ml)}}$$

Worm counts: The small intestine was collected from infected animals on d28 of infection, cut open and washed in PBS. The number of worms present in the intestinal lumen was then enumerated. Egg and worm counts were performed as per standard procedures (Camberis *et al.*, 2003).

2.3. Cell isolations

2.3.1 Leukocyte isolation from MLNs, spleen and liver

Single cell suspensions were prepared following protocols previously published by the MacDonald Laboratory (Phythian-Adams *et al.*, 2010) and as above in sections 2.1.2 and 2.1.3. For liver preparations, tissues were diced and digested for 45 min as described above. Liver leukocytes were isolated by centrifugation in 33% Percoll (GE Healthcare), followed by filtration through a 40µm cell strainer to remove contaminating *S. mansoni* eggs before RBC lysis. Single cell suspensions from all tissues were then prepared for flow analysis or cells (Liver and MLN: 1 x 10^6 cell/ml; spleen: 2 x 10^6 cell/ml) were cultured in X-vivo 15 medium (BioWhittaker) containing 2mM L-Glutamine and 50mM 2-ME (Invitrogen) in 96 well plates at 37°C in a humidified atmosphere of 5% CO_2 with or without 15µg/ml SEA. Supernatants were harvested from the cultures after 72h and cytokine production assessed by ELISA.

2.3.2 Small intestinal lamina propria cell isolation

The protocol used to isolate LP cells is based on protocols from the laboratories of Fiona Powrie and William Agace, and was adapted for helminth-infected guts by John Grainger (Belkaid Laboratory, NIH, USA). These adaptations include addition of dithiothreitol (DTT) to combat the high levels of mucus present in the intestine during helminth infection. The small intestine was isolated, and PP manually dissected. The gut was longitudinally slit open in ice cold 3% medium (RPMI-1640 (Sigma) plus 3% FCS (Sigma), 1% Penicillin/ Streptomycin (Gibco) and 20mM HEPES (Gibco)), and the intestinal contents was removed by gently scraping along the length of the gut. Gut was diced into Shake medium (RPMI-1640, plus 1% Penicillin/ Streptomycin, 20mM HEPES and 2mM EDTA). Samples were manually shaken for 30 seconds, after which contents was poured through a tea strainer (Sainsbury's), with liquid flow through discarded. Gut pieces were collected, and placed back into fresh Shake medium. The shake/draining process was repeated a further 2 times, after which intestinal pieces were placed into DTT medium (3% medium supplemented with 1% FCS, 0.5mM EDTA (Gibco) and 14.5µg/ml DTT (Sigma-Aldrich)). Samples were stirred on a Variomag magnetic stir plate (Thermo Scientific) for 15 min at 37 °C. Following stirring, the mixture was poured through tea strainers, and gut pieces placed back into fresh Shake medium. Samples were manually shaken/drained three times as described earlier, after which intestinal pieces were placed into digest medium (RPMI-1640, plus 1% Penicillin/ Streptomycin, 20mM HEPES, 1% L-Glutamine, 0.1% β-mercaptoethanol, 5mg DNase and 5mg Liberase). Intestines were further diced manually, and stirred on the magnetic stir plate for 24 min at 37 °C. Following stirring, digestion was stopped by adding 30 ml of 3% medium. Gut pieces were mashed through a 70µM, then a 40µM cell strainer in 3% medium. Cells were then resuspended in X-Vivo medium for restimulation or prepared for flow analysis.

2.3.3 T cell isolation for DC:T cell co-culture

Spleen, mesenteric, brachial and inguinal lymph nodes were pooled, crushed through gauze to release cells, centrifuged at 1200g for 5 minutes at room temperature, RBCs were lysed using RBC lysis buffer (Sigma), cells were washed and stained with CD4-APC (Table 2.1), prior to sorting using a BD Aria-II.

2.4 *In vitro* bone marrow DC differentiation

2.4.1 Bone marrow preparation

Tibias and fibulas of the hind legs of mice were collected into ice cold PBS (GMDCs) or complete RPMI (FLDCs), bones were then sterilised in 70% Ethanol (Sigma) for 3 minutes, before washing with PBS/ cRPMI, to remove all ethanol. Individual bones were selected, the ends of the bone were removed using a scalpel and 5ml of PBS/ cRPMI was injected through the bone using a 5ml syringe (BD) and a 21G needle (BD), flushing the bone marrow into a 50ml falcon tube (BD). Flushed bone marrow samples pooled from all bones collected were then disrupted by repeatedly passing through a 23G needle (BD). A sample of BM was mixed in a 1:1 dilution with trypan blue and the numbers of HSCs present were counted based on estimated size.

2.4.2 Heat-inactivation of foetal calf serum

The foetal calf serum serum (FCS) used in BMDC medium was placed at 58ºC for 50 minutes to heat inactivate it prior to use. Heat inactivation was used to denature the components of the complement cascade in the serum that could damage the cells being cultured or prepared.

2.4.3 Flt3-L BMDC culture

FLDCs were cultured essentially as in Naik *et al*. (Naik *et al.*, 2010), flushed BM was RBC lysed and 1.5 x 10^5/ml bone marrow cells were resuspended in complete Flt3-L medium (RPMI-1640 (Sigma) containing 10% FCS (Hyclone/Sigma), 2mM L-Glutamine (Gibco), 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen) 50mM 2-Mercaptoethanol (Invitrogen), plus 200ng/ml rFlt3-L (Peprotech). Flasks were cultured at 37°C in a humidified atmosphere of 5% CO2 for 8 days without feeding. On day 8 cells were harvested, and re-suspended in fresh medium containing 50ng/ml FLT3L for *in vitro* stimulations, see section 2.4.

Optimisation experiments were performed initially to select the optimal concentration for plating of BM cells for FLDC generation, 1.5 x 10^5/ml was found to yield the best returns and healthy cells. A number of different FCS batches from different sources were also tested prior to characterisation experiments. Hyclone was found to provide the highest yield of live, immature cells. However, during the project it was necessary to batch-test a

new source of FCS. Sigma was chosen on this occasion, based on cell yield, lowest activation status and cost. Thus there is some variability in cell culture methods, however, the cells grown from Hyclone and Sigma FCS were found to be relatively comparable.

2.4.4 GM-CSF BMDC culture

Bone marrow (BM)-derived dendritic cells (DC) were prepared following protocols previously published from the MacDonald laboratory (MacDonald *et al.*, 2001). 2 x 10^6 bone marrow cells were seeded in 10ml complete medium (RPMI-1640 (Sigma) containing 20ng/ml rGM-CSF (Peprotech), 10% FCS (Hyclone/Sigma), 2mM L-Glutamine (Gibco), 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen)). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. On day 3, 10ml of complete medium was added, on days 6 and 8, 9ml of media was gently (to avoid undesired activation of cells) aspirated and replaced with 10ml of fresh complete medium, cells were harvested gently on day 10. Immature d10 BMDCs were resuspended in fresh medium containing 5ng/ml GM-CSF for *in vitro* stimulations, see below (section 2.4).

Due to the sensitivity of BMDCs to movement and temperature, there is inherent variability in the basal activation status of the cells used in all BMDC experiments. This is particularly the case with GMDCs, which must be removed from the incubator during culture for feeding. The method to count HSCs in BM preparations is not precise and down to personal assessment of cell size, and as such there is also variability in the yield of cells after the differentiation period. Another confounding factor in the activation status of BMDCs is that FCS is not a uniform preparation due to its biological nature. To minimise the variation in BMDCs on account of FCS (and growth factors), we spend a considerable amount of time batch-testing from various companies to ensure we get comparable growth, and then bulk-buy the FCS, Flt3-L and GM-CSF batch that provides the highest yield, lowest activation status, at the lowest price. However, as outlined above, it is some times unavoidable that some changes are made to the reagents used.

2.5 *In vitro* stimulations and functional assays

2.5.1 Preparation of Ags

Propionibacterium acnes (Pa) is an anaerobic bacterium and was grown by the lab of Professor Ian R Poxton (Microbial Pathogenicity Research Laboratory, UoE) before being

heat-killed and stored under sterile conditions at 4ºC. Heat-killed *P. acnes* was aliquoted and the concentration of protein per ml was determined for each aliquot using a Bradford (coomassie) protein assay and a known BSA standard.

Attenuated *Salmonella typhimurium* strain SL3261 was supplied by Dr. Maurice Gallagher (UoE) was heat-killed and stored under sterile conditions at 4ºC prior to use. The heat-killed *S. typhimurium* was aliquoted and the concentration of protein per ml was determined for each aliquot using a Bradford (Coomassie) protein assay and a known BSA standard.

Eggs were purified from the livers of mice infected with 180 *S. mansoni* cercariae. Livers alone are used as a source of eggs to limit the amount of endotoxin contamination that may occur from using tissues of mice, it was felt that the bacterial load of intestines would be too high to make this tissue a viable source of eggs. Livers were harvested on d49 of infection from mice. C57BL/6 mice were used to avoid allogeneic responses following antigen challenge of C57BL/6 experimental mice with eggs or SEA purified and prepared from livers. Livers were washed in ethanol prior to being minced and then digested overnight at 37°C with 1.47U/ml Collagenase D, in the presence of Penicillin/ Streptomycin and Polymyxin B Sulphate, in an attempt to limit bacterial contamination. Digested livers were then washed twice before being mashed to break up any remaining liver fragments. Following a wash with PBS, the remaining pellet was resuspended in PBS and overlayed on a 33% Percoll gradient. The gradient was spun at 1400 RPM and the upper layers of the Percoll gradient removed to leave the eggs in a pellet at the bottom of the tube. Eggs were washed with PBS, counted and then frozen at -80°C. Soluble egg antigen from *S. mansoni* was prepared in-house as previously described (MacDonald *et al.*, 2001). The stored eggs were thawed and transferred to a Tenbroeck 7 ml tissue grinder to which 5 ml sterile PBS was added. The eggs were then carefully homogenised using a twisting and grinding motion. After ~20 rotations, egg disruption was checked. This was repeated ~15 times on ice until 95% of the eggs were disrupted. The homogenate was transferred to a 15 ml tube and spun at 2800 xg for 15 min at 4ºC. The supernatants were transferred to 1 ml micro-centrifuge tubes and spun at 16,000 xg for 10 min. The pooled supernatants were filter sterilised through a 0.45µm filter. The SEA protein concentration was determined using a Bradford (Coomassie) protein assay and a known BSA standard. The

SEA was aliquoted and stored at -80ºC.

SEA prepared in the lab has been tested using the Limulus assay to assess potential endotoxin contamination, which demonstrated that there is no activation of Limulus by our SEA, thus any LPS contamination is below the level of detection of this assay. Further to this, GMDCs are extremely sensitive to LPS and we do not see any surface activation or cytokine production from these cells when cultured with SEA. Any batch that did activate an inflammatory cytokine response from these cells would be immediately discarded. Since 2002, only one batch from the lab has needed to be discarded.

2.5.2 DC Exposure to Ag

BMDCs were cultured for 6 or 18h in the presence or absence of SEA (25µg/ml), Pa (10µg/ml), St (5µg/ml) or CpG-A (1µg/ml, ODN 1585 (Invivogen)). Ag concentrations were based on published studies (Jenkins *et al.*, 2007; Perona-Wright *et al.*, 2012), that had previously shown these concentrations to be effective for Th2 polarisation by BMDCs, in the case of SEA, or Th1/Th17 priming by BMDCs following exposure to St/Pa. 6h Ag exposure was used for gene expression analysis, and 18h culture for assessment of surface phenotype and cytokine production. For the timecourse experiment described in section 3.2.4, BMDCs were cultured in the presence or absence of Ag for between 3 and 24h. The results from the timecourse experiment were used to guide the length of time cells were cultured for gene expression analysis, as this experiment was used to evaluate gene expression at different timepoints, as well as surface phenotype and cytokine production. It was found that IFN-I genes were activated by SEA from 6h onwards.

2.5.3 DC: OT II co-cultures

This assay was set up following protocols from the MacDonald laboratory, as previously described (Cook *et al.*, 2012). For CFSE dilution assays $CD4^+$ OT II TCR Transgenic T cells were purified from spleen and LN using $CD4^+$ Dynabeads (Invitrogen) following the manufacturer's protocol. T cells were labelled with 5 µM CFSE (Invitrogen) for 15 min at 37 °C, excess CFSE was allowed to leach from the cells prior to culture with 5 x 10^4 WT or *Ifnar1*$^{-/-}$ BMDCs in the presence of 0.01µg/ml OVA323-339 or 5µg/ml OVA protein (Sigma) which had been endotoxin depleted in-house. Cultures were incubated at 37°C for 4 days prior to assessment of CFSE dilution by flow cytometry.

2.5.4 DC: T cell co-cultures

This assay was set up following protocols from the MacDonald laboratory, as previously described (Cook *et al.*, 2012). 50,000 IL-10eGFP$^-$ x KN2 CD4$^+$ or IL-13eGFP$^-$ x KN2 CD4$^+$ T cells were cultured in 96 well plates for 3d with 2,500 WT or *Ifnar1*$^{-/-}$ BMDCs, 1µg/ml anti-CD3 (produced in-house) and with or without IL-4 (20 ng/ml, Peprotech).

2.5.5 DC chemotaxis assay

The method used to assess DC chemotaxis using a transwell assay was based on that described by Wendland *et al*. (2011), and was adapted for BMDCs to measure chemotaxis following exposure to Ag. Optimisation experiments were performed to establish which serum-free medium could be used for the assay, whilst limiting any effects on cell health or maturation status. Optimisation experiments were also used to establish the number of BMDCs to use per well, so that enough cells would be present in the migrated population for thorough analysis of surface phenotype by flow cytometry. WT or *Ifnar1*$^{-/-}$ BMDCs were cultured overnight in the presence or absence of SEA as described in section 2.5.2. Cells were washed and resuspended in X-Vivo medium (Lonza). 400µl of medium containing 0, 25, 100, 250nM of CCL21 was added to the bottom chamber of a transwell plate. 1 x 10^6 cells in 100µl of medium were placed in the top chamber of each transwell. Cells were incubated at 37°C for 3h. After 3h, the medium in the bottom chamber was harvested, and the bottom of the transwell insert washed to remove any adhered cells. Cells were then washed and stained for flow analysis and the MACSQuant (Miltenyi) used to acquire flow data and calculate the number of each DC subset present per sample. The MACSQuant was used as this machine takes up a specific volume per sample and calculates a cell count in known volume to provide a total cell count that should be more accurate than any generated by BD machines.

2.5.6 TLR reporter cell assay

The TLR reporter assay was performed by members of Hermilijn Smits laboratory (LUMC, Leiden, Netherlands). HEK293-TLR3 and HEK293-CD14/TLR4 cells were maintained in DMEM culture medium, supplemented with 10% FCS, 10mg/ml ciprofloxacin and 5mg/ml puromycin. For stimulation, cells were seeded at 3.5 x 10^4 cells/well in 96-well flatbottom plates and were stimulated the next day. For stimulation of HEK-293-CD14/TLR4 cells,

12.5% supernatant of MD-2 transfected cells was added. IL-8 production was measured in supernatants after 22 hours using a commercial kit (Sanquin), as per the manufacturer's instructions.

2.6 Human samples

2.6.1 Human DC isolation and generation

Preparation and stimulation of human DCs was performed by Bart Everts (University of Washington, USA). PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from heparinised venous blood of healthy Western volunteers. $CD141^+$ DCs were purified from PBMCs using the BDCA3/ $CD141^+$ DC isolation kit (Miltenyi Biotech). $CD1c^+$ DCs were purified from the CD141-depleted PBMCs using the BDCA1/$CD1c^+$ DC isolation kit (Miltenyi Biotech). Subsequently, to maximize cell yield pDCs (BDCA4/$CD123^+$) were isolated from the flowthrough of the mDC isolation by using BDCA-4 Dendritic Cell Isolation Kit (Miltenyi Biotec).

Monocytes were isolated from the same healthy volunteers as above by $CD14^+$ MACS isolation (Miltenyi Biotec) and were cultured in RPMI medium supplemented with 10% FCS, 50ng/ml human rGM-CSF (Invitrogen), and 25U/ml human rIL-4 (R&D Systems). On day 3, culture medium including the supplements was replaced, and on day 6, immature mo-DCs were stimulated with the indicated Ags.

Freshly isolated DCs or mo-DCs (2×10^4 cells/well in 200µl) were cultured in complete RPMI-1640 medium containing 10% FCS, 100U/ml penicillin, and 100mg/ml streptomycin and supplemented with 500U/ml GM-CSF or 10ng/ml IL-3 (both Strathmann) for $CD1c^+$ mDCs and pDCs respectively. DCs were cultured in the presence or absence of SEA (50µg/ml), R848 (1µg/ml, Invivogen), CpG-B 2006 (1µg/ml, Invivogen) or pI:C (100µg/ml, Axxora). After 40h cells and supernatants were harvested.

2.6.2 Human serum samples

Venous blood was obtained from 36 people (mean age 32 years and 9 months) from a Kenyan cohort, living in an area in which *Schistosoma mansoni* infection is endemic. Infection with *S. mansoni* was determined by faecal egg counts, using the mean count of 2 slides counted from 3 consecutive samples (see Table 2.1). Preparation of serum samples

was performed by members of David Dunne's laboratory (University of Cambridge). See Table 2.1 for details of cohort.

2.7 Analytical techniques

2.7.1 Flow cytometry

For viability staining: Cells were surface-stained for CD11c and CD45R as detailed below (without the addition of Live/Dead). Cells were washed once in Annexin V buffer (Life Technologies) and then stained in 50µl of Annexin V buffer plus 7-AAD (Life Technologies) and Annexin V-PE (Life Technologies) at a 1:250 dilution for 10 min in the dark at room temperature. 150µl of buffer was then added and cells run directly on the FACS machine.

For surface staining: Cells were washed in PBS and stained with LIVE/DEAD Aqua or Blue (Invitrogen) at a 1:500 (Blue) or 1:1000 (Aqua) dilution in 10µl of PBS, for 10 min at room temperature. Surface markers and FcR block (αCD16/CD32, 2.4G2, produced in-house) were then added to cells in 50µl of FACS buffer, for 20 min at 4°C, at the concentrations indicated in Table 2.2.

For intracellular cytokine staining: For restimulation prior to intracellular cytokine staining, 2 x 10^6 cells were plated in a 96-well round bottom plate (Costar) and stimulated with 0.5µg/ml PMA (Sigma-Aldrich) and 1µg/ml Ionomycin (Sigma-Aldrich), with 10µg/ml Brefeldin A (Sigma-Aldrich) included for the 3h, at 37°C with 5% CO_2.

After restimulation with PMA/Ionomycin/Brefeldin, cells were stained with LIVE/DEAD and surface markers as described above. Cells were washed three times in FACS buffer, and fixed for 20 min at 4°C in Cytofix/Cytoperm (BD Bioscences). Following fixation, cells were washed three times in Perm/Wash (BD Biosciences) and stained with antibodies in 50µl of Perm/Wash (BD Biosciences) at the concentrations indicated in Table 2.5 for 20 min at 4 °C. Isotype matched control antibodies were used on a pooled sample. Foxp3 staining was performed using the eBioscience Foxp3 staining kit, as per the manufacturer's instructions. Marker expression was measured on FACSCanto (BD Biosciences) or LSRII (BD Biosciences) flow cytometers and data were analysed using FlowJo software (Tree Star).

2.7.2 Enzyme-linked immunosorbent assays (ELISAs)

Mouse cytokine ELISAs were performed on culture supernatants using paired mAb (See Table 2.3) purified in house, purchased from eBioscience, BD Pharmingen or PBL, and recombinant cytokine standards purchased from PeproTech or PBL. The TNFα ELISA was perfomed using a DuoSet ELISA development kit (R&D systems). Primary/capture antibodies were coated onto 96 well plates (NUNC) in a volume of 50µl in PBS overnight at room temperature (TNFα, IL-1β, IFNα, IFNβ) or 4°C (IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-5, IL-4, IL-17). Plates were blocked using 10% NCS in PBS for >1h. Supernatants and doubling dilutions of recombinant protein standards were added to 96 well plates in a volume of 50µl, in duplicate where possible, single wells were used in situations where supernatant or plate space was limiting. Secondary/detection antibodies were added in a volume of 50µl in 10% NCS/PBS, and allowed to bind for 1h at 37 °C (IL-4, IL-10, IL-13), 1h at room temperature (IL-12p40, IL-12p70, IL-5, IL-6, IL-17) or 2h at room temperature (TNFα, IL-1β, IFNα, IFNβ). Streptavidin-peroxidase was added to all plates, except for IFNα/β, in a volume of 50µl and incubated at 37°C for 30 min. 50µl horse-radish peroxidase-conjugated rabbit IgG (Bethyl) was added to IFNα/β plates for 1h at room temperature (1.7µg/ml). 100µl of the colorimetric substrate of peroxidase, TMB (Sigma), was added to each well, following development of blue colour, reaction was stopped by addition of 0.18M H_2SO_4 acid (Sigma). Between steps, plates were washed using PBS 0.05% Tween-20 (Sigma). See Table 2.3 for reagent details.

Human DC supernatants were analysed for IFNα production by ELISA. The ELISA was used as per the manufacturer's instructions (MabTech).

2.7.3 CBA

Cytokines present in sera or cell supernatants were assayed using a CBA flex set (BD Biosciences), using cytokine-conjugated beads and PE-detection reagent as per the manufacturer's instructions (Wendland *et al.*) or at 1/5 of the manufacturer's recommended concentrations (mouse). Samples were run on a Canto II or a FACSJazz (BD) and data were analysed in FlowJo (TreeStar).

Mouse:

Cytokine	Bead Position
IL-4	A7
IL-5	A6
IL-6	B4
IL-10	C4
IL-12p70	D7
IL-13	B8
IL-17A	C5
IFNγ	A4
TNF	C8

Human:

Cytokine	Bead Position
IL-1β	B4
IL-6	A7
IL-10	B7
IL-12p70	E5
IFNα	B8
TNF	C4

2.7.4 Microscopy

Sample preparation and microscopy analysis was performed by Jess Borger (UoE). For dsRed BMDC tracking experiments, lymph nodes were fixed in 4% PFA, incubated sequentially in 15% and 30% sucrose solutions overnight to quench residual PFA and then mounted in O.C.T embedding medium (Sakura). This was done to ensure that the dsRed signal was maintained. Thin section confocal microscopy was performed on 25 µm cryostat sections, which were fixed in ice cold acetone for 10 min, then incubated overnight with TCRβ and CD4 antibodies conjugated to fluorescein (eBioscience, UK) and CD45R (B220) antibody conjugated to Alexa647 (BD Biosciences, UK). Slides were washed extensively in PBS-T, 1x PBS then water and nuclei were stained with DAPI supplemented ProLong Fade Gold (Invitrogen) mounting media. Samples were imaged on the Leica SP5 II (Leica Microsystems) with lasers exciting with the 405, 488, 543 and 633nM laser lines and under the 40x and 63× objectives, using LAS AP software (Leica,

USA). Data was rendered and analysed using Volocity software (Improvision).

2.7.5 RNA isolation and qPCR

RNA was recovered from cells or tissues following suspension in the phenol-based product TRIzol (Invitrogen). Total RNA from liver tissue was isolated using the RNeasy Mini Kit (Qiagen).

For DC samples, phase separation of DNA/protein and RNA was performed using Chloroform. RNA was precipitated using 2-propanol, visualization of RNA pellet was enhanced by addition of glycogen (Ambion) during precipitation. 75% ethanol was used to wash RNA, prior to air-drying and resuspension in RNase free DEPC water (Ambion).

0.1-0.25µg RNA was used for the synthesis of cDNA using Superscript-III and oligo-dT (Invitrogen). Relative quantification was performed by qPCR analysis using the Roche Light Cycler 480, with LightCycler SYBR Green I master mix (Roche). Five serial 1:4 dilutions of a positive control sample of cDNA were used to create standard curves. Expression was normalised to the housekeeping genes (gene expressed constitutively for the maintenance of cellular function) glyceraldehyde3-phosphate dehydrogenase *Gapdh* (for DCs) or *Ubiquitin* (for tissue samples). For primer sequences used see Table 2.4. Rachel Lundie performed the RT-PCR analysis on liver samples.

2.8 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6 software. Student's T tests were employed to determine significant differences between sample groups. In chapter 3, Figure 3.2, ANOVA and Tukey's multiple comparison tests were used to identify statistical differences between groups. In situations where only two replicate wells or individual animals were used, statistical tests are not valid and as such have not been used. For correlation analyses, Pearson r tests were done on parametric data, and Spearman r tests on non-parametric data.

Table 2.1 Human Subjects

YOB = Year of birth; Sex: 0 = female, 1 = male; epg = Eggs/ g faeces

Colour code: Egg negative, Medium egg load, High egg load

ID	YOB	Sex	epg
022-08	1987	0	0.0
032-01	1960	0	0.0
051-02	1982	1	0.0
059-01	1967	1	0.0
069-02	1976	0	0.0
091-01	1968	1	0.0
110-01	1968	1	0.0
173-06	1990	1	0.0
173-07	1980	1	0.0
183-01	1972	0	0.0
222-07	1977	0	0.0
231-04	1989	1	0.0
005-01	1959	1	126.7
026-04	1989	0	113.3
060-03	1966	1	113.3
087-03	1991	0	120.0
106-05	1990	1	140.0
113-08	1984	0	110.0
114-01	1970	1	120.0
117-05	1981	0	106.7
123-03	1990	1	136.7
236-05	1973	0	100.0
002-06	1992	1	873.3
004-03	1993	0	1496.7
015-04	1991	1	613.3
025-04	1990	1	473.3
038-06	1990	1	513.3
109-04	1991	1	476.7
112-07	1969	1	630.0
120-03	1984	1	766.7
177-02	1969	0	246.7
178-01	1965	0	380.0
193-07	1984	1	483.3
218-06	1986	0	193.3
280-02	1988	1	466.7
290-07	1988	0	193.3

Table 2.2 Flow antibodies

Antigen	Clone	Isotype	Host	Conjugate	Titration	Manufacturer
CCR5	HM-CCR5	IgG	Ar Ham	AF488	1:200	eBioscience
CCR7	4B12	IgG2a κ	Rat	APC	1:50	Biolegend
huCD2	S5.5	IgG2a	Mouse	PE	1:200	Invitrogen
CD4	RM4-5	IgG2a κ	Rat	APC, AF700, FITC	1:800	eBioscience
CD8	53-6.7	IgG2a κ	Rat	PECy7	1:800	BioLegend
CD103	2E7	IgG	Ar Ham	Bio, FITC, PerCP/Cy5.5	1:50	eBioscience
CD11b	M1/70	IgG2b κ	Rat	FITC, AF780	1:600	BioLegend
CD11c	N418	IgG	Ar Ham	AF647, AF780, PE	1:200	eBioscience
CD19	1D3	IgG2a κ	Rat	AF450, PE, PECF594	1:200	BioLegend
CD24	M1/69	IgG2b κ	Rat	PECy7	1:1000	Biolegend
CD25	7D4	IgM κ	Rat	PerCP/Cy5.5	1:200	Biolegend
CD40	3/23	IgG2a κ	Rat	PE, FITC	1:200	BD Pharmingen
CD45	30-F11	IgG2b κ	Rat	AF700	1:400	eBioscience
CD45.1	A20	IgG2a κ	Mouse	FITC	1:200	eBioscience
CD45.2	104	IgG2a κ	Mouse	PE	1:100	
CD45R (B220)	RA3-6B2	IgG2a κ	Rat	AF450, APC, BV650	1:200	eBioscience
CD80	16-10A1	IgG2 κ	Ar Ham	APC, PE, PerCP/Cy5.5, BV605	1:600	BD Pharmingen
CD86	GL-1	IgG2a κ	Rat	FITC, BV650, PECy7	1:200	BD Pharmingen
F4/80	BM8	IgG2a	Rat	PECy7, FITC	1:400	BioLegend
Foxp3	FJK-16s	IgG2a κ	Rat	AF450	1:200	eBioscience
IFNAR1	MAR1-5A3	IgG1 κ	Mouse	PE	1:200	Biolegend
IL-4	11B11	IgG1	Rat	PE	1:200	BioLegend
IL-5	TRFK5	IgG1	Rat	PE	1:200	BD Pharmingen
IL-13	eBio13A	IgG1 κ	Rat	AF488	1:200	eBioscience
IFNγ	XMG1.2	IgG1 κ	Rat	AF450	1:200	eBioscience
Gr1 (Ly6C+Ly6G)	R86-8C5	IgG2b κ	Rat	PerCP/Cy5.5, BV570	1:400	BD Pharmingen
Ly6C	HK1.4	IgG2b	Rat	BV570	1:400	Biolegend
Ly6C	AL-21	IgM	Rat	APC	1:400	BD Pharmingen
Ly6G	1A8	IgG2a κ	Rat	AF780	1:200	BD Pharmingen
MHC II (I-A/ I-E)	M5114	IgG2b κ	Rat	PerCP/Cy5.5, FITC	1:1600	eBioscience
MHC II (I-A/ I-E)	M5115	IgG2b κ	Rat	AF450	1:800	eBioscience
Siglec F	E50-2440	IgG2a κ	Rat	PE	1:200	BD Pharmingen
Streptavidin				PerCP/Cy5.5	1:1000	eBioscience
TCRβ	MR5.2	IgG2a κ	Mouse	AF780	1:200	eBioscience

Table 2.3 ELISA reagents

Primary Antibodies					
Specificity	**Conjugation**	**Clone**	**Host**	**Concentration**	**Manufacturer**
IL-1β	Purified	B122	Ar Ham	4μg/ml	eBioscience
IL-4	Purified	11B11	Rat	2μg/ml	Homegrown
IL-5	Purified	TRFK5	Rat	2μg/ml	Homegrown
IL-6	Purified	MP5-20F3	Rat	2μg/ml	Homegrown
IL-10	Purified	JES16-E3	Rat	1μg/ml	eBioscience
IL-12 p40	Purified	C15.6	Rat	2μg/ml	eBioscience
IL12 p70	Purified	9A5	Rat	2μg/ml	BD
IL-13	Purified	Ebio13A	Rat	2μg/ml	eBioscience
IL-17	Purified	TC11-18H0	Rat	0.5μg/ml	BD
IFNα	Purified	RMMA-1	Rat	2.5μg/ml	PBL
IFNβ	Purified	RMMB-1	Rat	2.5μg/ml	PBL
IFNγ	Purified	R4-6A2	Rat	2μg/ml	Homegrown
TNFα	Purified	Duoset DY410		0.8μg/ml	R&D
Recombinant Standards					
IL-1β	Recombinant			20ng/ml	eBioscience
IL-4	Recombinant			20ng/ml	Peprotech
IL-5	Recombinant			20ng/ml	BD
IL-6	Recombinant			20ng/ml	Peprotech
IL-10	Recombinant			50ng/ml	BD Pharmingen
IL-12	Recombinant			20ng/ml	Peprotech
IL-13	Recombinant			50ng/ml	Peprotech
IL-17	Recombinant			20ng/ml	eBioscience
IFNα	Recombinant			20ng/ml	PBL
IFNβ	Recombinant			112ng/ml	PBL
IFNγ	Recombinant			50ng/ml	Peprotech
TNFα	Recombinant			0.4ng/ml	R&D
Secondary Antibodies (Biotinylated)					
IL-1β	Biotin	Polyclonal sera	Rabbit	0.3μg/ml	eBioscience
IL-4	Biotin	BVD6-24G2	Rat	0.25μg/ml	BD
IL-5	Biotin	TRFK4	Rat	0.3μg/ml	eBioscience
IL-6	Biotin	MP5-32C11	Rat	0.5μg/ml	eBioscience
IL-10	Biotin	SXC-1	Rat	0.2μg/ml	BD
IL-12/23 p40	Biotin	C17.8	Rat	0.2μg/ml	eBioscience
IL-13	Biotin	Polyclonal sera	Rabbit	0.1μg/ml	Peprotech
IL-17	Biotin	TC11-8H4.1	Rat	0.25μg/ml	BD
IFNα	Biotin	Polyclonal sera	Rabbit	0.78μg/ml	PBL
IFNβ	Biotin	Polyclonal sera	Rabbit	0.078μg/ml	PBL
IFNγ	Biotin	XMG1.2	Rat	0.2μg/ml	BD
TNFα	Biotin	Duoset DY410		0.2μg/ml	R&D

Table 2.4 Primer sequences

Gene name	Fwd/Rev	Sequence
Gapdh	Fwd	AATGTGTCCGTCGTGGATCT
	Rev	CCCAGCTCTCCCCATACATA
Ubiquitin	Fwd	TGGCTATTAATTATTCGGTCTGCAT
	Rev	GCAAGTGGCTAGAGTGCAGAGTAA
Ifit1	Fwd	TCTAAACAGGGCCTTGCAG
	Rev	GCAGAGCCCTTTTTGATAATGT
Ifit3	Fwd	TGAACTGCTCAGCCCACA
	Rev	TCCCGGTTGACCTCACTC
Mx1	Fwd	TTCAAGGATCACTCATACTTCAGC
	Rev	GGGAGGTGAGCTCCTCAGT
Cd40	Fwd	GAGTCAGACTAATGTCATCTGTGGTT
	Rev	ACCCCGAAAATGGTGATG
Cd80	Fwd	GGATACATGGTATTATGTGGCTCA
	Rev	TGCAGAGGCTTCACCTAGAGA
H2ab	Fwd	TTTTCATCCGTCACAGGAGTC
	Rev	GTCAAAACACTCTGAGTCACTGC
Ifna1	Fwd	GGAACAAGAGAGCCTTGACA
	Rev	GAGGGTTGTATTCCATGCAG
Ifnb	Fwd	GAACATTCGGAAATGTCAGG
	Rev	ACTGTCTGCTGGTGGAGTTC

3.0 FLT3-L DEPENDENT BMDC RESPONSES TO SCHISTOSOME EGG ANTIGENS

3.1 Introduction

The use of CD11c-depletable mice has revealed that DCs are essential for the generation of Th2 immunity in response to both helminth parasites and allergens (Hammad *et al.*, 2010; Phythian-Adams *et al.*, 2010; Smith *et al.*, 2012). There is an extensive literature examining how DCs respond to Th2-inducing pathogens, particularly the egg stage of *S. mansoni*, and this has primarily utilised murine BMDCs generated *in vitro* in response to GM-CSF. These studies have shown that Th2 induction requires DC expression of CD40 and NF-κB, whilst OX40L functions to amplify the response (Artis *et al.*, 2005; Jenkins *et al.*, 2007; MacDonald *et al.*, 2002c). In the case of CD40, *in vivo* infection experiments with gene-deficient mice support these conclusions (MacDonald *et al.*, 2002b; Straw *et al.*, 2003). This indicates that reductionist studies with *in vitro*-derived DCs can model and inform the more challenging task of assessing DC:T cell interactions in multiple tissues (the skin, lungs, liver, intestine, draining lymph nodes) that occur over the course of *S. mansoni* infection. Despite this, the nature of the polarising signal provided by DCs that instructs Th2 differentiation remains unknown, although DC secretion of the classical Th2 polarising cytokine IL-4, or the Th2 and regulatory cytokine IL-10, is not required (Jankovic *et al.*, 2004; MacDonald and Pearce, 2002; MacDonald *et al.*, 2001).

Addition of GM-CSF to bone marrow cultures leads to the generation of a homogenously $CD11c^{hi}$ MHC II^{+} $CD8\alpha^{-}$ cell population (Inaba *et al.*, 1992; Lutz *et al.*, 1999), that most closely resemble fully-differentiated monocyte-derived $CD11b^{+}$ DCs *in vivo* (Robbins *et al.*, 2008). GMDCs have been, and continue to be, a very useful primary DC model, as it is possible to generate large numbers of cells *in vitro* using GM-CSF. Another advantage of GMDCs is that they are a single DC subtype so there is no need to sort or purify subsets. However, GM-CSF does not govern DC development *in vivo* (Naik, 2008; Waskow *et al.*, 2008), thus it does not seem that GMDCs have any direct counterpart in mice in the steady state. It now seems more likely that GM-CSF is required for DC homeostasis and survival *in vivo* rather than as a DC growth factor (Greter *et al.*, 2012). Hence, GMDCs fail to represent the level of heterogeneity seen in the DC populations present in lymphoid organs, or in peripheral tissues in the steady state.

More recently, it has been demonstrated that murine BMDCs can be generated *in vitro* using Flt3-L (Brasel *et al.*, 2000; Brawand *et al.*, 2002), which is the primary DC differentiation factor *in vivo* (D'Amico and Wu, 2003; Kingston *et al.*, 2009; Waskow *et al.*, 2008). Unlike GMDCs, cDCs generated *in vitro* with Flt3-L have clear steady state equivalents *in vivo*. Flt3-L gives rise to two *in vitro* cDC subsets which have similar properties to the cDC populations found in the spleen (Brasel *et al.*, 2000; Naik *et al.*, 2005). Flt3-L also generates a subset of plasmacytoid DCs (pDCs), these cells share many characteristics with their *in vivo* counterparts (Brawand *et al.*, 2002; Naik *et al.*, 2005).

In contrast to the large number of studies using GMDCs, there are currently no published studies investigating how Flt3-L derived BMDCs (FLDCs) respond to either schistosome antigens, or those from other helminth species. Because of the dichotomy in the roles of these two growth factors in DC biology (differentiation vs. maintenance of DCs, steady state vs. inflammatory DC phenotype), we hypothesised that FLDCs would respond to helminth antigen in a manner distinct from GMDCs. As such, this first chapter will address how FLDCs respond to the strongly Th2-polarising egg-antigens from *S. mansoni*, and compare and contrast their responses to a Th1/17-polarising bacterial stimulus. We have performed a comprehensive analysis of the phenotype of FLDCs following exposure to SEA. This includes characterisation of the expression level of MHC II and co-stimulatory markers, molecules that are essential for T cell polarisation. We have also assessed the cytokine output of FLDCs in response to SEA. The findings from these studies have been surprising, with the cytokine response in particular providing novel insights into the responses of different DC subsets to SEA. This has led us to investigate the PRRs that are involved in SEA-sensing by DCs, and may facilitate important developments in our understanding of DC function in response to helminths.

3.2 Results

3.2.1 Identification of DC subsets in a Flt3-L bone marrow culture

As FLDC generation was a relatively new technique in the lab, we first sought to identify the DC subsets present in our Flt3-L BM cultures. The protocol used to generate FLDCs was based on that of Naik *et al*. (2010), who have published extensively on these cells (Naik *et al.*, 2005; Naik *et al.*, 2007; Xu *et al.*, 2007), and have shown that 8-9d culture of BM with Flt3-L generates fully-differentiated $CD11c^+$ cells with the functional characteristics of DCs (Naik *et al.*, 2005). BM cells were cultured with Flt3-L for 8d, before being replated and cultured for a further 18h (Fig. 3.1A). The presence of the different DC subsets was then analysed by flow cytometry. Over 95% of live-singlet cells were $CD11c^+$ (Fig. 3.1B). Within the $CD11c^+$ population, two distinct DC subpopulations could be identified by their differential expression of CD45R (Fig. 3.1B). The $CD45R^+$ pDCs comprised approximately 30% of the $CD11c^+$ population, however this did vary between cultures (20-45%). The $CD45R^-$ cDCs, which made up a greater proportion of the culture (50%), could be further subdivided into $CD11b^{hi}$ $CD24^{lo}$ ($CD11b^+$ cDCs) and $CD11b^{lo}$ $CD24^{hi}$ (equivalent to $CD8\alpha^+$ cDCs, referred to as $CD24^+$ cDCs from now on) populations (Fig. 3.1B). There was a small proportion, around 20%, of $CD11c^{hi}$ cells that were not clearly definable as $CD45R^+$ pDCs or $CD45R^-$ cDCs. They may be the *in vitro* equivalents of a cell population identified in the spleen that are a developmental stage of cDCs, these cells downregulate expression of CD45R during differentiation (Segura *et al.*, 2009). This may explain a minority of cells expressing intermediate levels of CD45R (Fig. 3.1B). Having confirmed the presence of pDCs, $CD24^+$ and $CD11b^+$ cDCs in our FLDC cultures, we next assessed their responses to Ag.

3.2.2 SEA stimulates an intermediate level of phenotypic activation in conventional FLDCs

To determine how FLDCs respond to helminth Ag, BM cells were differentiated with Flt3-L for 8 days and then cultured overnight in the presence or absence of SEA (Fig. 3.2A). A heat-killed preparation of the attenuated *Salmonella typhimurium* strain SL3261 (St) was used as a Th1/17-inducing control. Unless otherwise stated, these characterisation experiments use bulk FLDC cultures that are a mixture of the cDC subsets and pDCs. There is no doubt that it would have been preferable to sort the FLDCs into pure populations. However, this was technically impractical due to the amount of flow-sorting

that would have been required to obtain the numbers of cells needed for every experiment. We also found that flow sorting could have a detrimental impact on the survival and phenotype of our cells. In our initial experiments we focused on the surface phenotype of cDCs versus pDCs and did not breakdown our characterisation to include the different cDC subsets. This was primarily because of limitations in the number of markers we were able to analyse on our FACS machines at the time. More recently we have been able to extend our flow panel and have characterised the responses of the cDC subsets to SEA, which are detailed in section 3.2.7.

As expected, FL-cDCs upregulated expression of MHC II and the co-stimulatory molecules CD40, CD80, and CD86 in response to St (Fig. 3.2B), this was also evident in a significant increase in gMFI of these markers (Fig. 3.2C). FL-pDCs also upregulated these molecules when stimulated with St (Fig. 3.2B-C). However, their expression level of MHC II and CD40 was approximately 15 fold lower than St-stimulated cDCs, whilst that of CD80 and CD86 was 80 and 25 fold lower respectively. More tellingly, only CD86 was significantly upregulated on pDCs exposed to St, compared to medium alone (Fig. 3.2C). Furthermore, in the absence of any Ag stimulation, pDCs had a much lower basal level expression of these markers compared to cDCs (Fig. 3.2B-C).

From our previous work with GMDCs, we predicted that SEA would induce little to no change in FLDC activation phenotype (Jenkins *et al.*, 2007; MacDonald *et al.*, 2001; Perona-Wright *et al.*, 2006a). Surprisingly, however, SEA caused a marked increase in MHC II and co-stimulatory molecule expression, which was also evident in a significant increase in gMFI (Fig. 3.2B-C). In fact, MHC II expression levels on SEA and St-stimulated FL-cDCs were equivalent. FL-pDCs displayed no significant upregulation of expression of MHC II, CD40, CD80 or CD86 in response to SEA (Fig. 3.2B-C).

Thus SEA appeared capable of generating an intermediate level of FL-cDC phenotypic activation. This is in contrast with results previously obtained using GMDCs, which are essentially unresponsive to SEA (MacDonald *et al.*, 2001; Perona-Wright *et al.*, 2012). This finding led us to next investigate whether SEA exposure also affected cytokine production by FLDCs.

3.2.3 SEA stimulates IFN-I secretion from FLDCs

In order to assess FLDC cytokine production in response to SEA or St, ELISAs were performed on cell supernatants from cells cultured overnight with these Ags. In line with the marked phenotypic activation of FLDCs following exposure to St (Fig. 3.2), the bacterium also stimulated high-level secretion of the inflammatory cytokines IL-1β, IL-6, IL-12p40, IL-12p70 and TNFα (Fig. 3.3A), all mediators of Th1 and/or Th17 immunity (Kapsenberg, 2003; Muranski and Restifo, 2013). By contrast, SEA elicited much lower levels of these cytokines from FLDCs, although significant upregulation of IL-6 and IL-12p40 was noted when compared to medium alone controls (M, Fig. 3.3A). St also induced the secretion of IL-10 from FLDCs, which was not the case for SEA-pulsed cells (Fig. 3.3A).

In line with the initial identification of their *in vivo* counterparts as 'IFN-producing cells' (Perussia *et al.*, 1985; Trinchieri and Santoli, 1978), FL-pDCs produce large amounts of IFN-I in response to viral and synthetic TLR ligands (Brawand *et al.*, 2002; Naik *et al.*, 2005). FL-cDCs can also secrete IFNα following stimulation with bacterial or viral preparations, albeit at lower levels (Brawand *et al.*, 2002). Thus, we measured IFN-I production by these cells, and found they secreted high levels of IFNα3 and IFNβ in response to the TLR9 ligand, CpG ODN 1585 (Fig. 3.3C). Similarly, St also stimulated IFNα production by FLDCs (Fig. 3.3B), whereas IFNβ production was at a lower level and more variable, only being detectable on occasion (none detected in the experiment shown in Fig. 3.3B).

Whilst the response of these cells to bacterial and TLR agonists was as expected, we were extremely surprised to find that SEA was also a robust stimulus for IFN-I production (Fig. 3.3B) by FLDCs. SEA induced both IFNα3 (at a level similar to St) and IFNβ subtypes.

3.2.4 The kinetics of FLDC activation and IFN-I production in response to SEA

Since the above findings assessed the responses of FLDCs only after 18h of Ag stimulation, we next performed a timecourse study to determine the kinetics of the FLDC response. Bulk FLDCs were cultured with or without Ag and supernatants from triplicate wells were harvested after 3, 6, 12, 18 or 24h stimulation. Cells were also stained for

analysis of surface marker expression by flow cytometry at 3, 6, 18 and 24 h. For simplicity, Fig. 3.4A focuses on cDC expression of CD40 only at these timepoints. However, these data are also representative of the kinetics of CD80 and CD86 expression. Expression levels of these activation markers in FL-pDCs exposed to SEA/ St was negligible.

cDCs cultured in medium alone displayed a basal expression level (gMFI 300-400) of CD40 on their surface that was largely unchanged over a 24h period, and the total percentage of $CD40^+$ cells actually decreased over time (Fig. 3.4A). In stark contrast, at the earliest timepoint analysed (3h), essentially 100% of cDCs activated with St were already $CD40^+$, with a high level of CD40 expression on their surface (Fig. 3.4A). After 3h of SEA exposure, both the proportion of FL-cDCs that were $CD40^+$, and the level of CD40 expression, was increased compared to unstimulated cells. Despite this, SEA activated cDCs expressed almost 10-fold lower levels of CD40 than St exposed cells at this early timepoint (Fig. 3.4A). The proportion of cDCs expressing CD40 between 6 and 18h remained stable in the St cultures, with a stepwise increase in geoMFI at each timepoint (Fig. 3.4A). After 24h, however, the expression level of CD40 had started to drop on cells exposed to St, and there was a minor decrease in the proportion of cells expressing this marker. This suggests that the St response may be downregulated at later timepoints. The kinetics of the cDC response to SEA were somewhat different because the percentage and level of CD40 expression continued to rise between 6-24h, to the extent that by 18h >99% of cells were $CD40^+$, and remained so at the 24h time point (Fig. 3.4A). This suggests that, as SEA appears to stimulate a lower level of activation when compared to St, the response develops slower and may peak later.

Culture supernatants were collected over the timecourse for ELISA analysis of FLDC cytokine secretion levels. As with phenotypic activation, IFNα release in response to St was significantly above background at the earliest timepoint (3h, $p=0.0034$, Fig. 3.4B), and peaked between 6-9h. In this experiment, no IFNβ was detected in the supernatants of St-pulsed cells at any timepoint. Again, the response to SEA was slower. Significant but low levels of IFNα3 were detectable above background at 6h ($p=0.0008$), and IFNβ at 9h, following SEA exposure ($p=0.0147$, Fig. 3.4B). However, most SEA-specific IFN-I was secreted between 6-12h of Ag stimulation. Levels of St-induced IFNα and SEA-IFNβ

seemed to be reduced at later timepoints (Fig. 3.4B). Together, these data suggest that the kinetics of both the phenotypic activation and IFN-I response of FLDCs to SEA is different to that generated by bacterial exposure, with potential consequences for co-ordination of downstream T cell responses.

3.2.5 FLDCs generated from 3 different mouse strains have the same phenotypic and cytokine response to SEA

Using cells generated from C57BL/6 BM, we found that IFN-I was secreted in response to SEA stimulation by FLDCs, and that cDCs particularly became activated by exposure to this helminth Ag preparation. To address whether this is a general feature of the murine FLDC response to helminth Ag, the phenotype of FLDCs generated from the BM of two other commonly used laboratory mouse strains (BALB/c and CBA) was also assessed (Fig. 3.5A).

Both SEA-pulsed BALB/c and CBA FL-cDCs displayed the intermediate activation level previously seen with BL/6 cells, with the gMFI of the co-stimulatory molecules being highest following St stimulation, but SEA inducing significantly above-background levels (Fig. 3.5B). GMFI levels for MHC II were more variable between wells, and so less easily interpreted. There was no significant difference in the gMFI of MHC II or CD40 on SEA-pulsed cells from the 3 strains. However, BALB/c FL-cDCs expressed significantly more CD80 (BL/6 and CBA: $p<0.0001$) and CD86 (BL/6: $p=0.0003$, CBA: $p=0.0051$) than cells from the other strains (Fig. 3.5B). CBA expression of CD80 ($p=0.0042$) and CD86 ($p=0.0003$) was also significantly elvated compared to BL/6 cells exposed to SEA.

SEA stimulated significant IFNα production by FLDCs from all three mouse strains (Fig. 3.5C). The exact concentrations of IFNα3 secreted varied between strains, with a mean concentration of 0.55ng/ml from BL/6, 1.23ng/ml from BALB/c and 0.82ng/ml from CBA cells (Fig. 3.5C). Again, the BALB/c output was significantly higher than the other strains (BL/6: $p=0.0001$, CBA: $p=0.0031$), and CBA IFNα levels were also significantly above BL/6, in response to SEA ($p=0.0008$). In this experiment, the level of IFNα production from St-pulsed cells was much lower than SEA levels, and CBA secretion was around background. Together, these data indicate that the responses of FLDCs to SEA represent

a consistent phenotype across mouse strains. We next investigated whether any facets of this response were shared with GMDCs.

3.2.6 GMDCs phenotypic activation and IFN-I secretion does not occur in response to stimulation with schistosome egg Ag

As described in the introduction to this chapter, a large number of published studies have examined the responses of GMDCs to SEA, none of which have reported a marked phenotypic response from these cells. In agreement with this, we found significant upregulation of MHC II and co-stimulatory molecule expression on GMDCs only following overnight exposure to the bacterial stimuli St or Pa (the heat-killed gram-positive bacterium, *P. acnes*)(Fig. 3.6A-B). Similarly, no significant IFN-I secretion was detected in the supernatants of cells that had been exposed to SEA (Fig. 3.6C). These data indicate that, although capable of phenotypic activation and IFN-I production in response to bacterial stimulation, GMDCs do not respond to SEA in this way.

3.2.7 Differential activation of FL-cDC subsets by SEA

Thus far, this chapter has distinguished between the responses of cDCs and pDCs to SEA based on their surface expression of CD45R. However, as discussed in section 3.2.1, FL-cDCs are composed of both $CD11b^{hi}$ $CD24^{lo}$ and $CD11b^{lo}$ $CD24^{hi}$ cells, equivalent to *in vivo* $CD11b^{+}$ and $CD8a^{+}$ cDCs, respectively. For simplicity, the $CD11b^{hi}$ population will be referred to as $CD11b^{+}$ cDCs, and the $CD24^{hi}$ as $CD24^{+}$ cDCs. We assessed the response of the individual cDC subsets to SEA by FACS analysis of unsorted bulk FLDC cultures, and found clear differences in basal activation, as well as in the response to SEA. $CD11b^{+}$ cDCs expressed a significantly higher basal level of CD40 ($p<0.0001$) and CD80 ($p=0.0001$, Fig. 3.7A-B) than the $CD24^{+}$ subset. SEA-stimulated expression of these markers was also higher on $CD11b^{+}$ cDCs than $CD24^{+}$ cells, with gMFI for CD40 ($p=0.0199$) and CD80 ($p<0.0001$) 2 and 4-fold greater respectively than on the $CD24^{+}$ subset (Fig. 3.7B). It should be stated, however, that following SEA exposure, both these markers were significantly upregulated on the $CD24^{+}$ subset compared to unstimulated cells (CD40: $p=0.0007$; CD80: $p=0.0003$) (Fig. 3.7B). Moreover, whilst $CD11b^{+}$ cDCs upregulated CD80 to a greater extent than the $CD24^{+}$ subset in response to SEA, $CD24^{+}$ cDC expression of CD40 increased 4-fold in comparison to just a 2-fold increase on $CD11b^{+}$ cDCs (Fig. 3.7C). This suggests that both cDC subsets are responsive to SEA

stimulation but with some differences in their surface phenotype. There was no significant difference in MHC II expression between any groups in this experiment.

To determine the cellular source of the IFN-I produced by FLDCs in response to SEA, the three different FLDC subsets were FACS-sorted, stimulated for 18h with Ag, and then cell supernatants analysed for cytokine production. This revealed that only the $CD24^+$ cDCs produced significant levels of IFNα3 above background levels ($p<0.0001$, Fig. 3.7D). No IFNβ was detected from any cells in this experiment. Since neither pDCs nor $CD11b^+$ cDCs produced IFNα in response to SEA, this suggests that the receptor-signalling pathway involved in the IFN-I response is selectively expressed by $CD24^+$ cDCs over other subsets.

3.2.8 Receptor-signalling pathways involved in the FLDC IFN-I response to SEA

IFN-I production in response to pathogen Ags is mediated by PRRs, primarily by cytosolic nucleic acid sensors, such as RIG-like receptors (RLRs) or by transmembrane TLRs (Honda and Taniguchi, 2006a). To investigate which receptor-signalling pathways may be important in SEA-specific IFN-I induction by FLDCs, we began by addressing the role of TLRs. Two key adaptor proteins for relaying TLR-mediated signalling are MyD88 and TRIF, and so we assessed IFN-I production by *Myd88*$^{-/-}$/*Trif*$^{-/-}$ FLDCs, in response to SEA. This revealed that SEA induces IFNα3 in a MyD88/TRIF-dependent fashion (Fig. 3.8B). This finding suggested that RLRs were unlikely to play a central role in the IFN-I response, as a distinct adaptor molecule, IPS-1, mediates downstream signalling from RLRs (Kawai *et al.*, 2005).

To investigate the exact TLR responsible for this signalling, we measured responses in WT and TLR9-deficient FLDCs, since TLR9 (which recognises unmethylated CpG sequences in dsDNA and is MyD88-dependent) is the TLR most closely associated with IFN-I production by DCs (Kadowaki *et al.*, 2001a; Kadowaki *et al.*, 2001b). However, bulk cultures of *Tlr9*$^{-/-}$ FLDCs produced comparable concentrations of IFNα3 to WT cells ($p=0.1671$, Fig. 3.8C), indicating no major role for TLR9 in this case. Remarkably, MyD88-deficient, TRIF-sufficient cells showed a significant enhancement in SEA-specific IFNα3 compared to WT cells ($p=0.0003$, Fig. 3.8D), which clearly demonstrates that MyD88 is a negative regulator of the SEA-induced IFN-I response. Equally clear was the complete

ablation of IFNα secretion by *Trif*$^{-/-}$ FLDCs in response to SEA (p=0.0084, Fig. 3.8D), demonstrating that the IFN response is dependent on TRIF signalling. TRIF relays signals from TLR3 and TLR4 (Yamamoto *et al.*, 2003; Yamamoto *et al.*, 2002), suggesting involvement of one or both of these receptors in the IFN-I response to SEA. However, there are additional PRRs involved in the induction of this response, as *Ly75*$^{-/-}$ FLDCs (deficient in the CLR CD205) displayed a significant (p=0.0290), though only partial, reduction (40% of WT levels) in IFNα production after exposure to SEA (Fig. 3.8E). Together, these findings indicate that SEA interaction with FLDCs stimulates a complex, multi-faceted signalling cascade, one of the downstream outcomes of which is SEA-specific IFN-I production.

In order to determine whether SEA contains a TLR3 ligand, we tested for binding to a number of TLR reporter cell lines, in collaboration with Hermelijn Smits (LUMC, Netherlands). TLR3 activation by SEA was only 2-fold above medium background levels (demonstrated by IL-8 induction), this was approximately 6-10 fold lower than that induced by the known TLR3 ligand polyI:C (Fig. 3.9). Perhaps most tellingly, there was no discernible increase in TLR3 activation with increasing dose of SEA (Fig. 3.9). The TLR4-reporter cell line illustrates that there is a level of activation of TLR4 by SEA. However, TLR4 activation was downmodulated at a high dose of SEA, with a reduction in TLR4 activation at 50μg/ml compared to 10μg/ml (Fig. 3.9). Thus, more work is required to identify the exact PRRs that contribute to SEA-specific IFN-I production, as well as the receptors that mediate FLDC activation in response to SEA.

3.2.9 Summary:

- FL-cDCs displayed an intermediate level of surface activation in response to SEA, which includes a marked upregulation in MHC II expression and co-stimulatory molecules (Fig. 3.2), although not to the level of classical activation seen in response to a bacterial stimulus
- FL-pDCs responded to Ag stimulation with only very minor changes in the markers associated with activation (Fig. 3.2)
- The FLDC response to SEA was characterised by an almost complete lack of cytokine production (Fig. 3.3A), with the exception of IFN-I (Fig. 3.3B)
- Both $CD11b^+$ and $CD24^+$ FL-cDCs were activated by SEA, but $CD24^+$ cDCs were the source of SEA-specific IFN-I (Fig. 3.7)
- In agreement with published reports, GMDCs did not become phenotypically activated in response to SEA, nor did they secrete IFN-I in response to this helminth Ag (Fig. 3.6)
- The SEA IFN-I response was dependent on both TRIF and DEC205, but was negatively regulated by MyD88 (Fig. 3.8)

3.3 Discussion

The aim of the work detailed in this chapter was to address how Flt3-L generated BMDCs respond to SEA, the highly Th2-polarising egg Ags from the parasitic helminth *S. mansoni*. Although Flt3-L is the primary DC growth factor *in vivo*, most of the *in vitro* studies investigating DC responses to SEA have focused on GMDCs. Up to this point, the phenotype of FLDCs following exposure to helminth Ag had not been described. The DCs differentiated *in vitro* by Flt3-L are comprised of subsets that are equivalent to those found in lymphoid organs in the steady state, thus providing a model with which to study DC responses to SEA that is more relevant to the *in vivo* setting. This work demonstrated that FL-cDCs upregulated the markers associated with T cell priming following exposure to SEA, an intermediate level of surface activation in comparison to a bacterial stimulus. pDCs expressed only very low-levels of MHC II and co-stimulatory molecules but did display significant upregulation of CD40 and CD86 in response to SEA. Although the cytokine output from FLDCs in response to SEA was very low, we have identified that the $CD24^+$ FL-cDC subset uniquely produced IFN-I following exposure to SEA. This IFN-I response was dependent on the PRR adaptor protein TRIF, and optimal IFN-I output also required FLDC expression of the CLR CD205. In contrast, we failed to detect any IFN-I production by GMDCs exposed to SEA. Thus, the responses of FLDCs to SEA are somewhat distinct from those of GMDCs.

3.3.1 FLDC activation by SEA

In agreement with previous work (MacDonald and Maizels, 2008; MacDonald *et al.*, 2001), we found that C57BL/6 GMDCs fail to respond to SEA phenotypically with little change in surface expression of MHC II, CD40, CD80 or CD86 compared to unstimulated cells. In complete contrast, our studies with Flt3-L *in vitro* differentiated cDCs have indicated that SEA can stimulate an intermediate level of activation, as evidenced by a significant increase in MHC II, and a minor elevation in co-stimulatory molecule expression (Fig. 3.2B-C). Nevertheless, activation levels remain low in SEA exposed FLDCs, compared to those 'classically' activated by bacterial stimulation (Fig. 3.10).

As highlighted in the introduction to this chapter, FLDCs have been lauded as a model of DCs present in steady state lymphoid organs (Naik *et al.*, 2005; Naik *et al.*, 2007), suggesting that their responses to SEA *in vitro* may be representative of the phenotype of

their *in vivo* counterparts. A study by Straw *et al*. (2003) is, to date, the only published study that has tried to characterise the activation phenotype of DCs *in vivo* during patent *S. mansoni* infection. This paper analysed the surface expression of MHC II, CD40, CD80 and CD86 on MACS-purified $CD11c^+$ cells from the spleens of mice at different time points during infection. This $CD11c^+$ cell population in the spleen would likely contain resident $CD8\alpha^+$ and $CD8\alpha^-$ cDCs (equivalent to the $CD24^+$ and $CD11b^+$ cDCs in the FLDC culture respectively (Naik *et al.*, 2005)), as well as pDCs (Naik *et al.*, 2007). Whilst the authors saw an increase in MHC II, CD40 and CD80 expression over the course of infection on all $CD11c^+$ cells, this did not rise significantly above naïve levels until a relatively late timepoint (days 49-56), and CD86 expression did not vary significantly at any timepoint of infection (Straw *et al.*, 2003).

Our findings from FLDCs stimulated with SEA suggest we would expect to see DC activation around the commencement of egg deposition (from around d28 post-infection), but Straw *et al*. (2003) did not detect this. One possible explanation is that their initial analysis of splenic DCs included all $CD11c^+$ cells within the spleen, and did not separate the DC subsets. As we, and others (Asselin-Paturel *et al.*, 2001; Brawand *et al.*, 2002), have demonstrated, pDCs express only very low levels of MHC II and co-stimulatory molecules, which may influence interpretation of the readout for bulk $CD11c^+$ cells *ex vivo*. In unpublished work from our lab (Rachel Lundie *et al.*), we have assessed the activation status of cDCs and pDCs in different tissues over the course of *S. mansoni* infection. This work has indicated that DC responses to infection are quite distinct in different tissues, with splenic cDCs only displaying a significant increase in CD40 expression compared to naïve animals around d56 of infection. However, we have not completed a comprehensive analysis of the activation of the different cDC subsets.

Straw *et al*. (2003) did assess the activation status of $CD8\alpha^+$ and $CD8\alpha^-$ splenic cDCs during *S. mansoni* infection. In contrast to our findings with the FLDC subsets, where we saw a higher basal level of surface activation on $CD11b^+$ cDCs (the $CD8\alpha^-$ equivalents)(Fig. 3.7A-B), a greater proportion of the splenic $CD8\alpha^+$ cDCs from naïve mice expressed CD40, CD80 and CD86. Straw *et al*. found that the $CD8\alpha^-$ subset expressed only MHC II at a higher level than the $CD8\alpha^+$ cDCs in the spleen of naïve animals. On d56 of infection the $CD8\alpha^-$ subset displayed a greater fold increase in CD80 expression

compared to the CD8α$^+$ subset, whilst CD40 upregulation was more pronounced on CD8α$^+$ cDCs (Straw *et al.*, 2003). These findings are in agreement with our analysis of the responses of the CD24$^+$ and CD11b$^+$ FL-cDC subsets to SEA stimulation (Fig. 3.7C). However, the proportion of splenic cDCs that had upregulated MHC II and co-stimulatory molecule expression at this timepoint of *S. mansoni* infection seemed lower than is seen from FL-cDCs exposed to SEA *in vitro*.

Low to moderate DC activation in the spleen may be due to low-level Ag exposure in this tissue, compared to FL-cDCs in the *in vitro* setting. Similarly, sustained exposure to schistosome Ags in the spleen may only occur at later timepoints of infection when substantial tissue damage has been caused in the liver and intestine. We have termed the response of FL-cDCs to SEA an "intermediate activation phenotype", and this may be too subtle to be clearly detectable *in vivo* during infection. It is also worth noting that our studies of FLDCs *in vitro* examine the responses of these cells in isolation, whilst the *in vivo* setting is within a much more complex environment, including cell populations that regulate DC activation. During *S. mansoni* infection in particular, a large component of the immune response is regulatory, including an increase in Tregs, and activation of IL-10-producing Teffectors and innate cell populations (Dewals *et al.*, 2010; Herbert *et al.*, 2008; Hesse *et al.*, 2004). The response of splenic DCs to synchronous delivery of schistosome Ag, without the additional complexity of active infection, will be addressed directly in chapter 5.

Straw *et al*. (2003) restricted their analysis of DC phenotype to the spleen. However, the tissues that are predominantly affected by *S. mansoni* infection are the liver and intestine, and their dLN. This is because the immune response is primarily directed at parasite eggs, which become lodged in the liver and traverse the intestinal wall, causing severe damage (Wynn *et al.*, 2004). For this reason, it may be more applicable to compare FLDC activation to the phenotype of DCs from these tissues during *S. mansoni* infection. In agreement with our findings with FL-cDCs, data from our lab indicates that cDCs in the liver significantly upregulate their expression of CD40 from d28 of infection onwards (Rachel Lundie *et al*., unpublished data). Not only that, hepatic pDCs also upregulate expression of co-stimulatory molecules during infection. This agrees with our data from

SEA-pulsed FL-pDCs, which do become activated by SEA. However, both *in vitro* and *in vivo* pDCs express much lower levels of these markers than cDCs.

Our data raises the question of why Flt3-L and GM-CSF-dependent DCs respond so differently to SEA. One possible explanation is that GMDCs have a higher basal level of activation or maturation prior to exposure to helminth Ag, and this masks the intermediate level of activation induced by such stimuli. By contrast, this is readily detectable in the less mature FL-cDCs. In support of this, GMDCs have a higher granularity and size compared to FLDCs, and they also produce much larger concentrations of IL-12, TNFα and IL-10 in response to bacterial stimulation (Xu *et al.*, 2007), consistent with a more activated phenotype. However, it is not the case that GMDCs have a higher basal level of expression of MHC II or the co-stimulatory molecules, with the exception of CD80 (Fig. 3.2B-C and Fig. 3.6A-B), as has been shown in a published comparison of GMDCs and FLDCs (Xu *et al.*, 2007). Thus, it seems more likely that the differences we see in the response of GMDCs and FLDCs to SEA are more related to differential expression of receptor-signalling pathways, and the activation of particular pathways triggered by GM-CSF itself.

Ligation of the GM-CSF receptor leads to the activation of STAT3, STAT5 and NFκB (Ebner *et al.*, 2003; Feldman *et al.*, 1997; Laouar *et al.*, 2003). NFκB activation in particular is essential for effective APC function, regulating MHC II and co-stimulatory molecule expression (Yoshimura *et al.*, 2001). Ligation of Flt3, however, does not directly activate NFκB (van de Laar *et al.*, 2012), whilst STAT3 is essential for DC differentiation in response to Flt3-L (Laouar *et al.*, 2003; Onai *et al.*, 2006). STAT3 has been shown to interact with NFκB, leading to inhibition of NFκB-dependent responses, such as iNOS production (Yu *et al.*, 2002). Thus, it is possible to speculate that, in the absence of Ag stimulation, NFκB is largely inactive in FLDCs. NFκB expression is essential for optimal Th2 induction following SEA stimulation of GMDCs (Artis *et al.*, 2005), however, NFκB activation was not detectable in GMDCs at early timepoints after exposure to SEA (Kane *et al.*, 2004). This suggests that GM-CSF-dependent activation of NFκB enables effective SEA-specific Th2 polarisation by GMDCs. NFκB activation is tightly controlled, with NFκB itself initiating transcription of the negative regulator IκBα, which binds to NFκB, dissociating it from DNA and returning it to the cytoplasm in an inactive form (Natoli and

Chiocca, 2008). Gene transcription by NFκB is also terminated by degradation of NFκB subunits by ubiquitin ligases (Natoli and Chiocca, 2008). Thus, it could be that GM-CSF activation of NFκB renders this pathway unresponsive in GMDCs following exposure to Ags, such as SEA, that stimulate only low-level activation of signalling pathways. For this reason, we see no discernible change in GMDC surface phenotype in response to SEA. In contrast, it is reasonable to speculate that NFκB is activated in FLDCs following SEA exposure, leading to an upregulation in MHC II and co-stimulatory molecules. This could be assessed by Western blot analysis for NFκB subunits following SEA stimulation of FLDCs.

One way to definitively address the differences between FLDCs and GMDCs would be to perform in-depth gene expression analysis with a microarray-based approach. A study that looked at the effect of SEA on gene expression in GMDCs indicated that only 29 genes were significantly altered by SEA, in comparison to 551 by LPS (Kane *et al.*, 2004). Our lab has also carried out microarray analysis of SEA-stimulated GMDCs, which revealed that only a small number of genes were up- or downregulated in response to SEA (Peter Cook *et al.*, manuscript submitted). Microarray analysis of mRNA expression by FLDC subsets would allow us to confirm the changes we have seen at the level of cytokine secretion and surface protein upregulation, and also allow a more broad assessment of other significant changes in FLDCs that are induced by SEA.

Intriguingly, analysis of the responses of FLDCs from 3 different mouse strains suggests that the mouse strain, BALB/c, may be more responsive to SEA than cells generated from C57BL/6 or CBA BM (Fig. 3.5). Early studies of the immune responses of different mouse strains suggested that BALB/c mice had a more “pro-Th2” phenotype (Locksley *et al.*, 1999). However, there are a number of factors involved in this dominant Th2 phenotype, including a defect in Th1 induction in BALB/c mice, as well as an imbalance in Treg homeostasis (Sacks and Anderson, 2004). Studies of strain differences in response to infection with the gastrointestinal helminth *H. polygyrus* demonstrate that there are a number of genetic polymorphisms that influence the immune response in BALB/c mice, which include MHC II haplotype (Reynolds *et al.*, 2012). Thus, it is difficult to discern exactly why there are differences in the responsiveness of FLDCs from different mouse strains to SEA. However, it is clear that an intermediate level of surface activation and IFN-

I production in response to SEA are traits common to FLDCs from the three mouse strains tested.

A timecourse analysis of FLDC phenotype and cytokine production following SEA or St stimulation revealed that the development of the FLDC response to SEA (and potentially other helminth and Th2-polarising Ags) was slower to develop, but may be more prolonged, than that induced by the bacterium St (Fig. 3.4). DC phenotypic responses to St stimulation were already well developed after a 3h culture, whilst SEA-exposed cells did not show a comparative level of activation until after 18-24h (Fig. 3.4A). In agreement with these findings, it has been shown (albeit with GMDCs) that LPS stimulation leads to phosphorylation of the MAPK family members p38, JNK and ERK within 10min, and NFκB activation after 30min of Ag exposure (Kane *et al.*, 2004). In contrast, SEA-pulsed GMDCs displayed only transient and low-level activation of p38 and ERK, and no discernible effect on JNK or NFκB within the 60min period analysed. These findings suggest that ligation of TLRs by bacterial Ags, such as the TLR4 ligand LPS, which is expressed by the gram-negative bacterium *S. typhimurium*, leads to rapid and intense activation of downstream signalling pathways. This leads to changes in DC phenotype within 3h of Ag stimulation (Fig. 3.4A). In contrast, the downstream effects of SEA exposure seem to be slower to influence DC phenotype, and the activation of signalling pathways more subtle (Artis *et al.*, 2005). The slower kinetics in FLDC maturation in response to SEA stimulation may mean that the induction of Ag-specific T cell activation occurs later. Migratory responses of FLDCs to SEA and St will be assessed in the next chapter, however, from this data it may be hypothesised that migration of SEA-stimulated DCs would also be delayed compared to St-pulsed cells, with two distinct waves of DC migration from periphery to LN, fast induced by St, and slow induced by helminth Ag.

A facet of DC activation that the timecourse experiment in Fig. 3.4 does not address is how long cell responses are maintained following a more transient Ag activation, given that it is possible that exposure to Ag *in vivo* would be more short-lived. This could be addressed with a shorter Ag pulse, followed by washing and replating of cells for a period of time prior to assessment of their phenotype. However, during infection with helminths, such as *S. mansoni*, parasites are constantly releasing antigenic material, whether in the

form of excretory/ secretory products or eggs; thus, it is entirely possible that DCs in affected tissues will be exposed to Ag continuously or for prolonged periods.

3.3.2 The FLDC IFN-I response to SEA: A complex receptor-signalling pathway?

The IFN-I signature we have detected in FLDCs responding to SEA is not an entirely novel finding; earlier studies by Trottein *et al.* described an IFN-I response by DCs to live schistosome eggs. Their 2004 study analysed the gene expression profiles of D1 cells (an immortalised splenic DC cell line (Winzler *et al.*, 1997)) in response to schistosome eggs or larval Ags (SLA). They described an "inflammatory" gene expression profile from DCs exposed to schistosome eggs that was not seen in response to SLA (Trottein *et al.*, 2004). This profile included the upregulation of a number of IFN-responsive genes, and an increase in IFNβ mRNA, although the authors did not demonstrate egg-specific secretion of IFN-I protein from D1 cells. These findings were regarded as controversial at the time, with many in the field suggesting LPS contamination of the egg preparation a probable cause. Such an explanation was supported by the abnormally high production of the inflammatory cytokines IL-12/23p40 and TNFα by D1 cells in response to egg stimulation (Trottein *et al.*, 2004). Based on results described in this chapter, we would now argue that, whilst this excessive production of IL-12/23p40 and TNFα may be a response peculiar to this DC cell line, our work with FLDCs suggests that an IFN-I signature is a reproducible response to schistosome egg Ag by DC subsets that are equivalent to those resident in lymphoid organs.

An additional area for discussion is the description by Trottein *et al.* of an IFN-I signature as "inflammatory". IFN-I is a cytokine that has pleiotropic effects *in vivo*, whilst capable of enhancing inflammatory responses in some settings, high concentrations of IFN-I act to limit IL-12, and consequently IFNγ, production (Trinchieri, 2010). It has also been suggested that low-level IFN-I signalling is integral to maintaining function of a wide range of immune cells (Gough *et al.*, 2012). A study analysing the gene expression profile of D1 cells to different stimuli indicated that IFNα treatment actually stimulates a gene expression profile more consistent with an anti-inflammatory phenotype (Torri *et al.*, 2010). IFN-I undoubtedly has a central role in activating innate and adaptive immune responses to viral and bacterial infection (Fitzgerald-Bocarsly and Feng, 2007; Gonzalez-Navajas *et al.*, 2012; MacMicking, 2012). However, a number of studies have also highlighted an

important role for IFN-I in the regulation and augmentation of DC function. For example, IFN-I is required specifically by $CD8\alpha^+$ cDCs for induction of functional anti-tumour responses from $CD8^+$ T cells (Diamond *et al.*, 2011; Fuertes *et al.*, 2011). It has been suggested that both splenic and BMDCs (GMDCs) express IFN-I message basally, and that they require IFN-I responsiveness for optimal activation and function (Kurche *et al.*, 2012; Montoya *et al.*, 2002). A role for IFNα has also been described in mediating optimal DC turnover *in vivo* (Mattei *et al.*, 2009).

Furthermore, it is an oversimplification to suggest all IFN-I function in the same "inflammatory" manner, as they represent such a large and diverse protein family with both mouse and humans having 12 different IFNα subtypes, alongside IFNβ, IFNε, IFNκ and IFNω (Pestka *et al.*, 2004). In human PBMC-derived myeloid cells different cell types respond to specific TLR ligands, such as CpG and pI:C, with a cell-specific IFNα subtype expression profile (Hillyer *et al.*, 2012). Currently our understanding of the SEA-specific IFN-I profile from FLDCs is restricted to IFNα1 (at the mRNA level), and IFNα3 and IFNβ (protein secretion). All IFN-I subsets bind at the same site on their shared receptor, IFNAR, forming a complex of similar structure (Piehler *et al.*, 2012). However, differential IFN-I activities are determined by the lifetime and binding affinity of each subset (Piehler *et al.*, 2012). More thorough gene expression analysis is required to address whether other IFN-I subtypes are activated by SEA, which may provide insights into their role in helminth infection.

No mouse is currently available that has been genetically altered to be deficient in all 16 IFN-I genes, and the likelihood of a degree of functional redundancy means that single deficiency may be compensated for by other family members. Although there are no single deficiency mice currently available for IFNα, *Ifnb*$^{-/-}$ animals have been generated (Erlandsson *et al.*, 1998). The authors of this study examined the IFN-I response of IFNβ-deficient mouse embryo fibroblasts (MEFs) following viral challenge, these cells were unable to produce IFNβ *or* IFNα in response to Sendai virus infection, illustrating the importance of the IFNβ signal for IFNα induction. Thus, it would be informative to investigate the response of IFNβ-deficient FLDCs to SEA stimulation, to assess whether IFNα production by $CD24^+$ cDCs was also ablated. As an alternative approach, many studies into IFN-I function utilise *Ifnar1*$^{-/-}$ mice to elucidate the role of IFN-I. The IFN-I

receptor, IFNAR, is a heterodimer made up of IFNAR1 and IFNAR2 subunits and deletion of the *Ifnar1* gene leads to a loss of function and a failure of all IFN-I (α and β) to activate downstream effector functions (Hwang *et al.*, 1995). Mouse and human DC subsets express IFNAR (Diamond *et al.*, 2011; Severa *et al.*, 2006), and so are responsive to IFN-I. Study of IFNAR-deficient DCs indicates that IFN-I can act on DCs in an autocrine manner (Montoya *et al.*, 2002), which may explain aspects of our data that would otherwise be puzzling. In the timecourse experiment it seemed that concentrations of both IFNα and IFNβ secreted in response to Ag stimulation were reduced after longer culture times, and no IFNβ was detected from FLDCs exposed to St (Fig. 3.4B). It is likely that this is the result of uptake of IFN-I by cultured cells.

Assessment of IFNα production by cells selectively lacking the adaptor proteins MyD88 and TRIF indicate contrasting roles for these molecules in the SEA-specific IFN-I response (Fig. 3.8D) (Fig. 3.11). The requirement for TRIF for IFN-I production by FLDCs may suggest a role for TLR3 or TLR4, which can signal through this adaptor (Yamamoto *et al.*, 2002). TRIF (TIR-domain-containing adaptor-inducing interferon-β) was originally characterised for its role in the activation of IFNβ downstream of TLR3 (Yamamoto *et al.*, 2003; Yamamoto *et al.*, 2002). Two kinases have been identified which phosphorylate the TFs IRF3 and 7 in a TRIF-dependent manner, leading to transcription of IFN-I genes: IKKε and TBK-1 (Fitzgerald *et al.*, 2003; Sato *et al.*, 2003; Sharma *et al.*, 2003). IRF3/ 7 homodimers and IRF3-IRF7 heterodimers activate transcription of a large number of genes, including the genes for IFN-I subtypes (Honda and Taniguchi, 2006b). The role of this IRF-dependent pathway in the FLDC response to SEA could be definitively shown using *Irf3*$^{-/-}$ and *Irf7*$^{-/-}$ BM to generate DCs.

It is clear that MyD88 is not required for the induction of SEA-induced IFN-I, but instead actively inhibits this process (Fig. 3.8D). This phenomenon has been previously reported in other, non-helminth settings, demonstrating that MyD88 can negatively-regulate pathways downstream of TLR3 activation by at least two distinct mechanisms. Exacerbation of TRIF-dependent inflammation, initiated by pI:C treatment of mouse corneas in MyD88-deficient mice highlighted that MyD88 negatively regulates TLR3/TRIF signalling and subsequent CCL5 release by downregulating the activity of the MAP kinase, JNK (Johnson *et al.*, 2008). The increase in CCL5 output in MyD88-deficient animals led to increased infiltration

of inflammatory cells. A more recent study indicated that MyD88 could also inhibit phosphorylation and activation of IKKε, (but not TBK-1) which limits TRIF-dependent IRF3 activation and resultant CCL5 and IFNβ production following pI:C treatment of BMMΦs (Siednienko *et al.*, 2011). This inhibition is specific to TLR3 signalling and does not affect LPS-induced TLR4-dependent pathways (Siednienko *et al.*, 2011), which gives further weight to our hypothesis that SEA-specific IFN-I occurs via TLR3 and not TLR4. These studies also highlight that production of the leukocyte chemoattractant, CCL5, by SEA-pulsed FLDCs should be investigated.

Despite our hypothesis that the IFN-I response is TLR3-mediated, there is little evidence from a TLR3 reporter cell line that this receptor is strongly activated by SEA. The IL-8 production in response to SEA was much lower than was stimulated by the synthetic TLR3 ligand, pI:C (Fig. 3.9). This may reflect differences in the amount of stimulus added. It should also be noted that, whilst pI:C is a pure TLR3 ligand, SEA is a complex mixture of Ags, the majority of which are proteins, and the majority of those being heavily glycosylated (Jang-Lee *et al.*, 2007; Mathieson and Wilson, 2010). There is likely to be a much smaller nucleic acid content, only some of which may bind TLR3. Thus a fairer comparison would require a titration of pI:C concentrations, as well as a comprehensive assessment of the nucleic acids present in SEA.

The reporter cell line results also indicated a degree of TLR4 activation by SEA (Fig. 3.9). This may in part be due to low-level LPS contamination, as SEA is prepared from eggs purified from the livers of *S. mansoni* infected mice. However, SEA induces only very low-level secretion of IL-12, IL-6 and TNFα (Fig. 3.3A), cytokines associated with LPS stimulation, indicating that any LPS present in SEA has a minimal effect on these aspects of FLDC activation. Further, the absence of an SEA IFN-I response from GMDCs (Fig. 3.6C) indicates that IFN-I induction is unlikely to be down to LPS stimulation of TLR4, as GMDCs express high levels of this receptor and respond readily to LPS stimulation (Boonstra *et al.*, 2003). LPS contamination could be quantified definitively using the limulus amoebocyte lysate (LAL) assay (Young *et al.*, 1972), however, our BMDC studies should provide a reliable readout of LPS contamination, as we would expect to see surface activation and cytokine production if endotoxin was present in our SEA. The presence of an egg-derived TLR4 ligand should not be ruled out, however. Studies of the rodent filarial

nematode, *Acanthocheilonema viteae,* have identified ES-62, a glycoprotein present in parasite excretory/ secretory products, which binds TLR4 (Goodridge *et al.*, 2005). Moreover, a glycan, lacto-*N*-fucopentaose III (LNFPIII), present in SEA acts via TLR4 (Thomas *et al.*, 2003). GMDCs exposed to LNFPIII stimulated IL-4 production from TCR Tg OTII T cells, and this response was absent with TLR-deficient GMDCs. This highlights that TLR4 ligands exist in SEA and initiate Th2 induction. Thus, analysis of TLR3- and TLR4-deficient FLDC responses is crucial, in order to identify which TLR is the inducer of SEA IFN-I. RNase treatment of SEA prior to FLDC stimulation may also help determine whether this response is dependent on SEA-derived dsRNA ligands, which are recognised by TLR3. In order to selectively target dsRNA ligands present in SEA it necessary to use an RNase that acts specifically on dsRNA, such as a member of the RNase III family (MacRae and Doudna, 2007).

A published study has previously identified dsRNA ligands from live schistosome eggs that activate TLR3 (Aksoy *et al.*, 2005). The authors suggested two possible reasons for the presence of dsRNA in parasite eggs. Transcriptomic analysis of the different lifecycle stages of *S. mansoni* indicates that the parasite expresses genes involved in RNA interference, which creates dsRNA as a method of post-transcriptional gene silencing (Verjovski-Almeida *et al.*, 2003). Aksoy *et al.* (2005) also indicated that the schistosome genome contains large numbers of transposons, which are both transcribed and show secondary structure that could include formation of double-stranded RNA.

A number of papers have identified microRNAs in *S. mansoni* that may have a role in controlling parasite gene expression (de Souza Gomes *et al.*, 2011; Simoes *et al.*, 2011), and it may be these that were identified as schistosome dsRNAs in earlier studies. MiRNAs are short, non-coding RNAs that bind to messenger RNAs, providing post-transcriptional regulation of genes. It has been demonstrated that miRNAs can act as ligands for murine TLR7 and human TLR8, which recognise single-stranded RNA (Fabbri *et al.*, 2012). MiRNAs are formed from dsRNA precursors, and mature miRNAs bind to complementary sequences in target mRNAs (Winter *et al.*, 2009). The cellular machinery that is involved in RNAi also plays a role in the processing of miRNAs, and these proteins have been identified in adult schistosomes and eggs (Gomes *et al.*, 2009; Krautz-Peterson and Skelly, 2008; Verjovski-Almeida *et al.*, 2003). The ability of schistosomes to perform

RNAi has not been definitively shown. However, a number of studies have identified miRNAs in the *S. mansoni* genome, as well as their likely gene targets (de Souza Gomes *et al.*, 2011; Gomes *et al.*, 2009; Simoes *et al.*, 2011), suggesting that the parasite utilises this pathway to regulate gene expression (Cheng and Jin, 2012). It has not yet been investigated whether pre-microRNAs or mRNA-bound miRNAs could provide ligands for host TLR3 activation. However, they should be considered as potential dsRNA ligands that could be present in schistosome egg preparations.

Another TRIF-dependent PRR that recognises dsRNA has recently been identified. This sensor is a complex made up of the RNA helicases DDX1, DDX21, and DHX36, and is localised within the cytosol (Zhang *et al.*, 2011a). Similarly to our observations of the SEA response, stimulation of this receptor by pI:C led to low-level IFN-I secretion. Involvement of this receptor in the induction of SEA-specific IFN-I could explain the failure of SEA to activate TLR3. However, this receptor complex was identified in GMDCs (Zhang *et al.*, 2011a), which is in conflict with our finding that these cells are unable to produce IFN-I in response to SEA. As yet it has not been demonstrated that this RNA helicase complex is active in other DC subsets. Thus, analysis of TLR-deficient FLDC IFN-I production following SEA exposure is essential to identify which TRIF-dependent PRR is required for this response.

SEA may also be inducing IFN-I through a TRIF-independent pathway, in support of which we have shown that CD205 has a partial role (Fig. 3.8E). A recent study has indicated that signalling downstream of another C-type lectin receptor, Dectin-1, can activate IFN-I production from DCs, this process being dependent on the adaptor protein, Syk (Del Fresno *et al.*, 2013). The induction of IFNβ was also dependent on IRF5, whilst IRF3 and IRF7 had no role. This suggests that CLR ligation could initiate IFN-I production directly in response to SEA. The importance of these receptors in SEA-sensing has already been demonstrated (Everts *et al.*, 2012; Meevissen *et al.*, 2010; Meevissen *et al.*, 2011). The role of this pathway in IFN-I induction downstream of SEA stimulation could be investigated using *Irf3*$^{-/-}$, *Irf5*$^{-/-}$, *Irf7*$^{-/-}$ and *Syk*$^{-/-}$ BMDCs, as demonstrated by del Fresno *et al*. (2013).

If TRIF-dependent IFN-I activation is mediated by TLR3, the question remains how these ligands are made available to FLDCs *in vitro*. It has been shown that a potent Th2-polarising component of SEA is omega-1 (Everts *et al.*, 2009; Steinfelder *et al.*, 2009), a glycoprotein whose activity is dependent on both its glycan components, which permit cellular uptake via MR, and its RNase activity, which causes the degradation of host mRNA and subsequent inhibition of protein synthesis (Everts *et al.*, 2012). Although we do not yet know whether omega-1 or MR are involved in the IFN-I response of FLDCs to SEA, it is possible that there are other RNases in SEA, whose uptake is mediated by CD205, which could release SEA-derived dsRNA fragments within cells that could then act as TLR3 ligands (Fig. 3.11). It is also possible that SEA-derived RNases could generate dsRNA ligands from host cell-intrinsic nucleic acids. However, dsRNAs are not a feature of mammalian gene expression, nor are mammalian cells capable of RNAi, so it may be unlikely that dsRNA self-ligands could be generated by RNase activity. The potential requirement for RNases in the generation of ligands, and the dependency on CLRs to transport SEA-derived RNases into DCs (Everts *et al.*, 2012), may explain why negligible TLR3 activation was recorded by direct SEA stimulation of TLR3-transfected cells (Fig. 3.9). Alternatively, it has been shown that RNA polymerase III can convert DNA to dsRNA ligands that can be recognised by RIG-I, leading to IFN-I production (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). It is possible that schistosome egg DNA could be converted to a ligand that is recognised by a TRIF-dependent PRR. If this mechanism does occur, it seems unlikely that TLR3 would be involved, given that this receptor is localised to endosomes, whilst RNA polymerase III is active in the cytosol (Chiu *et al.*, 2009).

The Th2-promoting activity of omega-1 was found to be MyD88/TRIF-independent (Steinfelder *et al.*, 2009), however this study looked only at GMDC responses to this SEA-derived glycoprotein. We plan to address how FLDCs respond to omega-1 whether it is capable of inducing IFN-I from FLDC subsets, and whether Th2 induction by omega-1 stimulated FLDCs requires IFN-I signalling.

As well as its strongly Th2-polarising characteristics, omega-1 was also shown to inhibit LPS-induced IL-12/23p40 and p70 production (Everts *et al.*, 2009; Steinfelder *et al.*, 2009). The ability of SEA to inhibit bacterial-induced IL-12 has been well documented (Cervi *et al.*, 2004; Kane *et al.*, 2004; Kane *et al.*, 2008), and shown to be independent of TLR2,

TLR4 and MyD88 (Kane *et al.*, 2008). This process is almost wholly dependent on omega-1 (Everts *et al.*, 2009) and may be mediated via its ability to inhibit protein synthesis (Everts *et al.*, 2012). Alternatively, omega-1 may induce IRF3 activation, which can then bind directly to the IL-12b promoter, preventing IRF5 binding and inhibiting gene transcription (Koshiba *et al.*, 2013; Negishi *et al.*, 2012), These published studies focus on IRF3 activation downstream of RLRs rather than TRIF-mediated IRF3 activity, and the role of RLRs in SEA-sensing has yet to be assessed. However, it is possible that this may also occur following TRIF-induced activation of IRF3. The role of IRF3 in the control of bacterial-stimulated IL-12 from FLDCs and GMDCs should be assessed using IRF3-deficient cells.

Infection of *Tlr3*$^{-/-}$ mice with *S. mansoni* has revealed that the receptor is not involved in control of infection or pathology (Vanhoutte *et al.*, 2008). There was no alteration in parasite burdens or granuloma size on d49 of infection. Moreover, there was an enhancement of Th2 cytokine output from splenocytes from *Tlr3*$^{-/-}$ *S. mansoni*-infected mice, following αCD3 stimulation. This may suggest that TLR3-dependent DC-derived IFN-I plays only a minor role in patent infection. Alternatively, we would suggest that the ability of DCs to respond to IFN-I produced by other cellular sources during infection might be more important than their own ability to produce it in a TLR3-dependent manner. The importance of DC IFN-I responsiveness will be addressed in chapters 4 and 5.

3.3.3 Distinct responses to SEA from different DC subsets

A potential role for TLR3 in mediating the DC IFN-I production in response to SEA is in agreement with our discovery that SEA-specific IFN-I is produced exclusively by the CD24^{+} cDC subset (Fig. 3.7D and Fig. 3.10). It has been shown that CD24^{+} cDCs, the CD8α^{+}-equivalents, are the only population of FLDCs that express TLR3 at the mRNA and protein level (Jelinek *et al.*, 2011; Naik *et al.*, 2005). These papers also illustrated that TLR3 expression *in vivo* is restricted to the CD8α^{+} cDC subset and the closely related CD103^{+} DCs. CD24^{+} cDCs do express TLR4, albeit at a lower level than TLR3, (Naik *et al.*, 2005). Further to this, GMDCs have only negligible expression levels of TLR3 receptor (Jelinek *et al.*, 2011), providing a plausible explanation for why these cells do not secrete IFN-I in response to SEA, but do so following bacterial stimulation (Fig. 3.6C). It has also been shown that STAT5 activation downstream of the GM-CSF receptor inhibits IRF7

expression (Esashi *et al.*, 2008), which is essential for sustained IFN-I production, particularly IFNα (Honda *et al.*, 2005a). This provides a further physiological explanation for the paucity of IFN-I production by Ag-stimulated GMDCs.

It should be noted that CD205 is selectively expressed on $CD8\alpha^+$ cDCs *in vivo* (Vremec *et al.*, 2000), in agreement with its involvement in the SEA IFN-I response, which was unique to $CD24^+$ FL-cDCs. Thus, it would be useful to confirm that, of the FLDC subsets, $CD24^+$ cDCs alone express CD205. Due to the fact that the $CD11b^+$ FL-cDC subset did not produce IFN-I in response to SEA, but did become activated (Fig. 3.7A-B), it seems likely that TLR3 activation is not uniquely required for the upregulation of MHC II and co-stimulatory molecules in response to SEA. We do not yet know, however, why $CD11b^+$ cDCs have a higher basal level of expression of activation markers compared to $CD24^+$ cDCs. This may again relate to gene expression differences, which would be revealed by mRNA profiling.

One potential criticism of the experimental approach taken in this chapter is that, in keeping with the vast majority of the referenced studies using FLDCs, we have determined the response of mixed cultures of DC subsets, rather than analysing the individual subsets in isolation. As such, we cannot exclude the possibility that the presence of one subset may alter the response of another. Nevertheless, it remains possible that DC subset crosstalk may also occur *in vivo*. Multiple attempts at FACS sorting FLDCs revealed that the technique led to unacceptably high cell death (particularly for pDCs), an increase in basal activation levels, and often a loss of detectable IFN secretion. Because of this, we decided to limit pre-sorting to experiments that require only low cell numbers, such as the in vitro functional assays that will be discussed in the next chapter. Sorting was also performed to ascertain the cellular source of SEA-stimulated IFN-I (Fig. 3.7D). We would like to be able to verify the result of this experiment using ICC. However, because IFN-I specific ICC has poor sensitivity, and the amounts of IFN-I we are hoping to detect are quite low, this proved technically difficult.

In conclusion, data in this chapter reveals that schistosome egg antigens induce the cDC subsets generated by Flt3-L to display an intermediate surface activation phenotype. This phenotype is distinct from that displayed by GM-CSF-derived cells. We have found that

$CD24^+$ cDCs have a unique ability to produce IFN-I in response to schistosome egg Ags, and this process is dependent on TRIF-signalling and negatively regulated by MyD88. Having defined this unexpected phenotype, we then went on to address the T cell priming ability of FLDCs and the potential role of IFN-I in this process, which will be the topic of the next chapter.

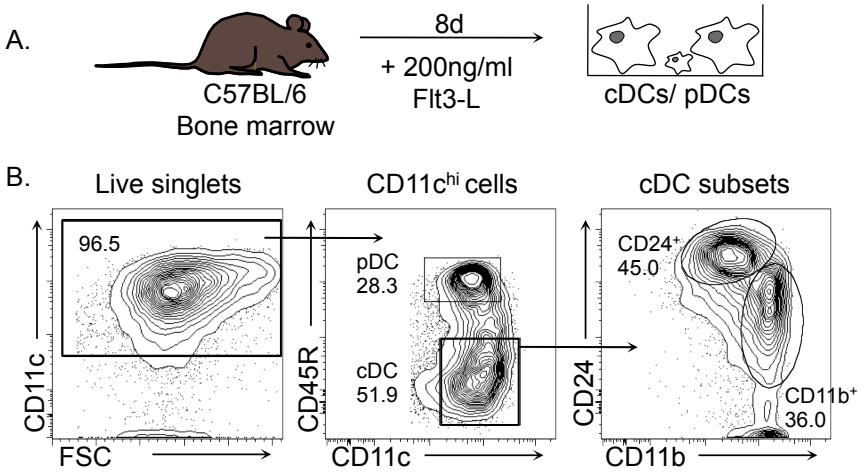


Figure 3.1 BMDC subsets generated *in vitro* using murine Flt3-L. BM cells were cultured for 8d with 200ng/ml Flt3-L before being re-plated on d8 and cultured overnight at a final concentration of $2x10^6$ cells/ml. On d9, DC subsets present in the culture were analysed by flow cytometry (A-B). Data representative of >5 experiments.

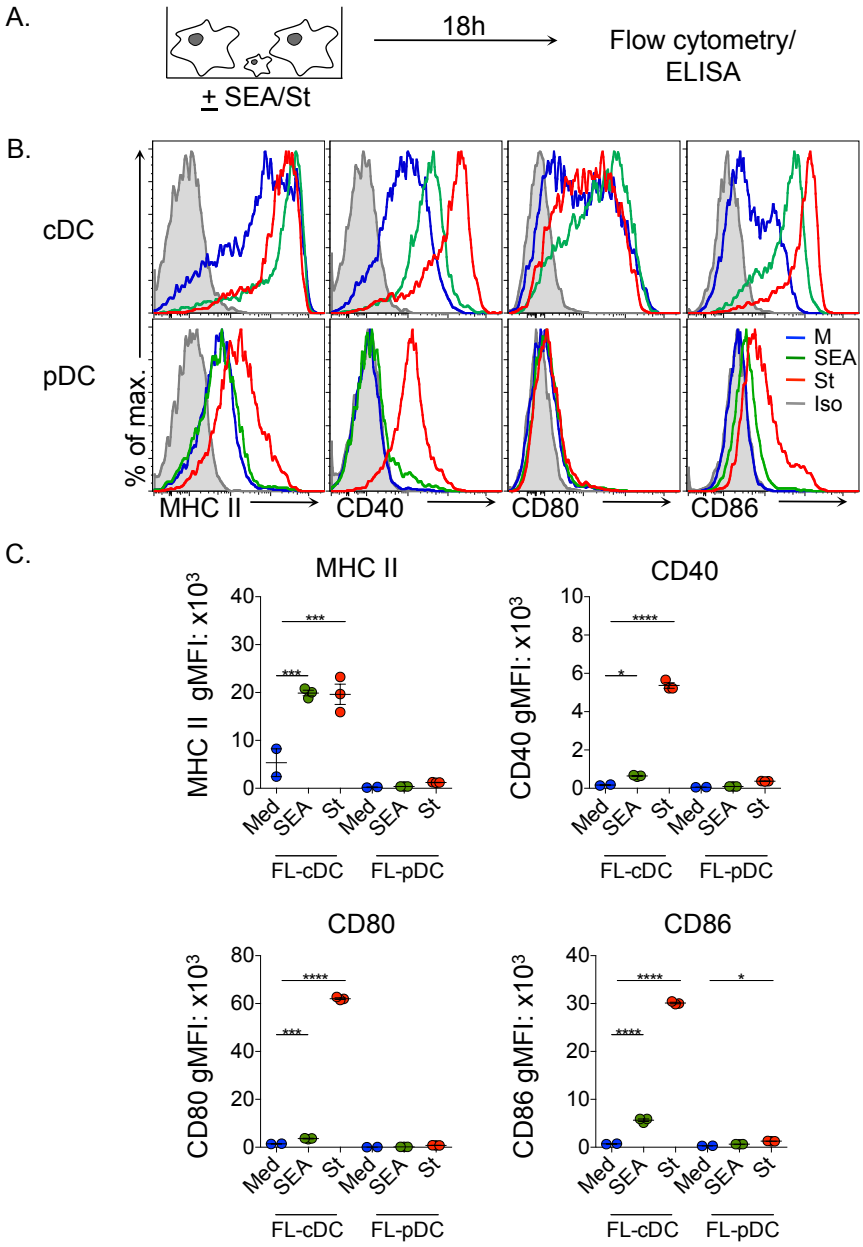


Figure 3.2 SEA stimulation induces an intermediate level of phenotypic activation in FL-cDCs.
FLDC cultures were stimulated for 18h with 25µg/ml SEA or 5µg/ml St and their expression of MHC II, CD40, CD80 and CD86 assessed by flow cytometry (B), gMFI of these markers was also assessed (C). Data representative of >5 experiments, performed in triplicate. Statistical differences calculated using ANOVA and Tukey multiple comparisons test. *P<0.05, ***P<0.001, ****P<0.0001.

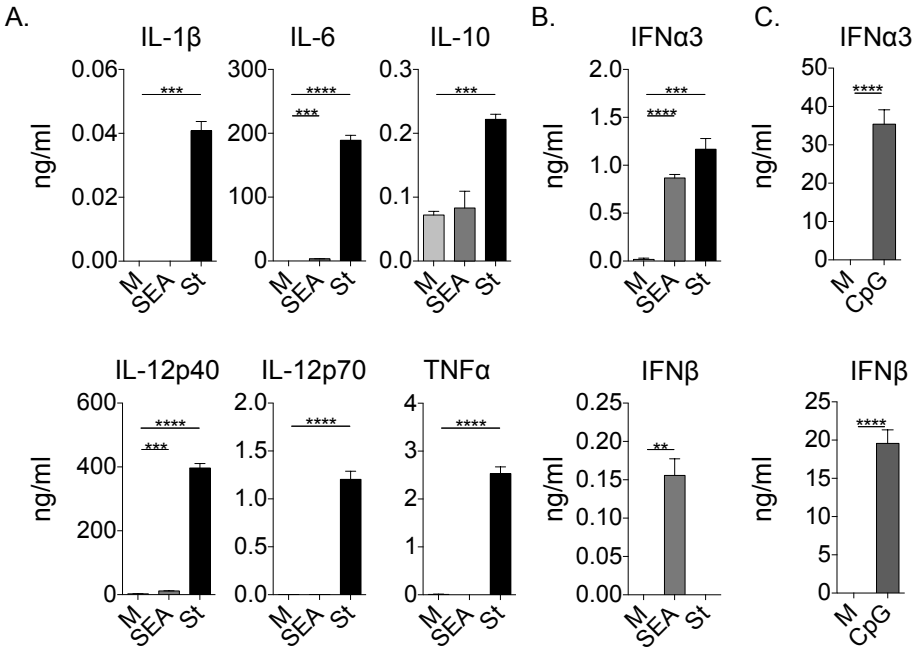


Figure 3.3 FLDCs secrete significant levels of Type I IFN in response to SEA stimulation, with background levels of inflammatory cytokines.
Bulk FLDC cultures were stimulated for 18h with 25μg/ml SEA, 5μg/ml St or 1μg/ml CpG, and cytokine levels in supernatants assessed by ELISA (A-C). Data representative of >5 experiments, performed in triplicate. **P<0.01, ***P<0.001, ****P<0.0001.

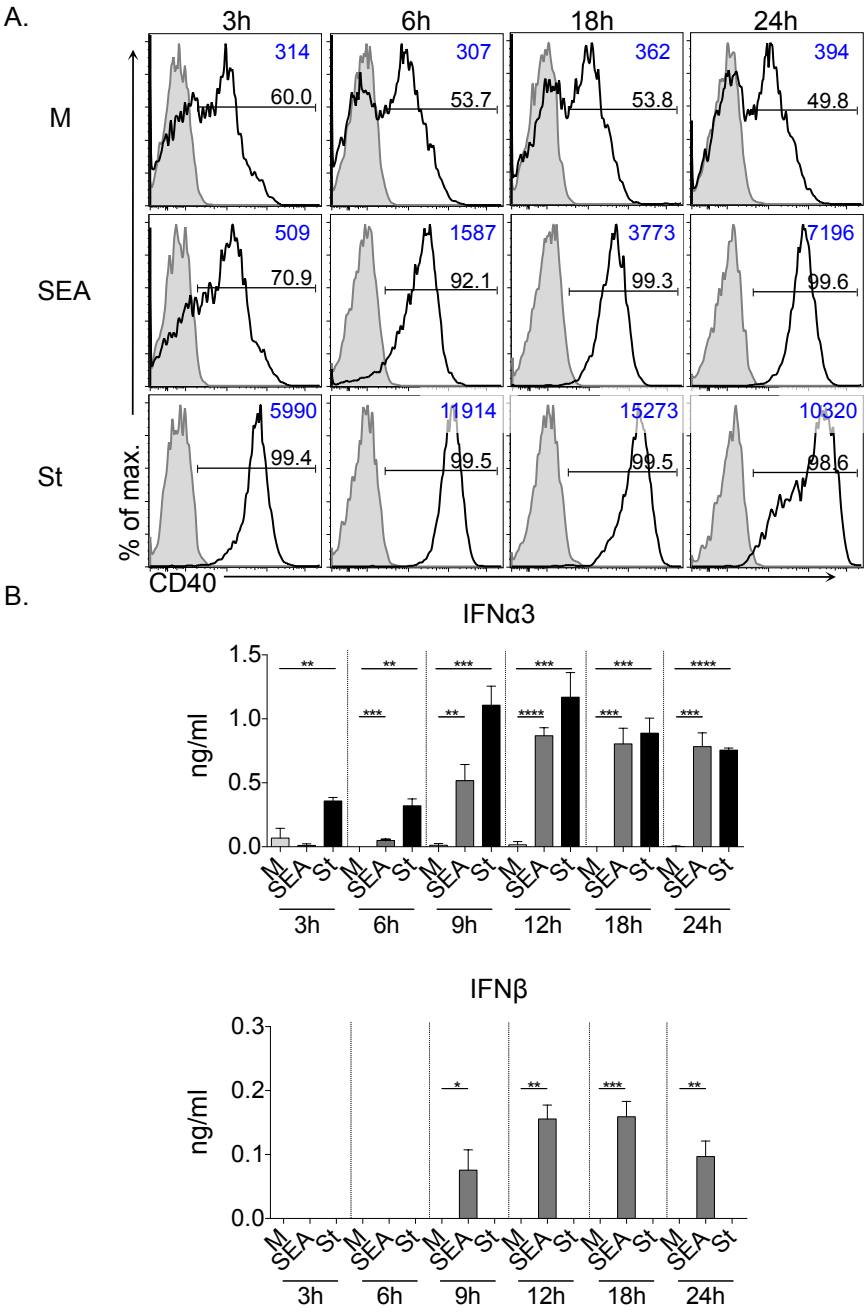


Figure 3.4 The kinetics of FLDC responses to SEA.
Mixed FLDC cultures were stimulated for 3-24h with 25ng/ml SEA or 5ug/ml St, phenotypic activation of cDCs was assessed by flow cytometry (A) and cytokine production measured by ELISA (B). Values in blue on plots equate to gMFI of CD40 expression, gate and corresponding value represents percentage of positive cells (A). One representative plot shown, of 3 replicate wells (A). Data representative of 2 independent experiments, done in triplicate. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

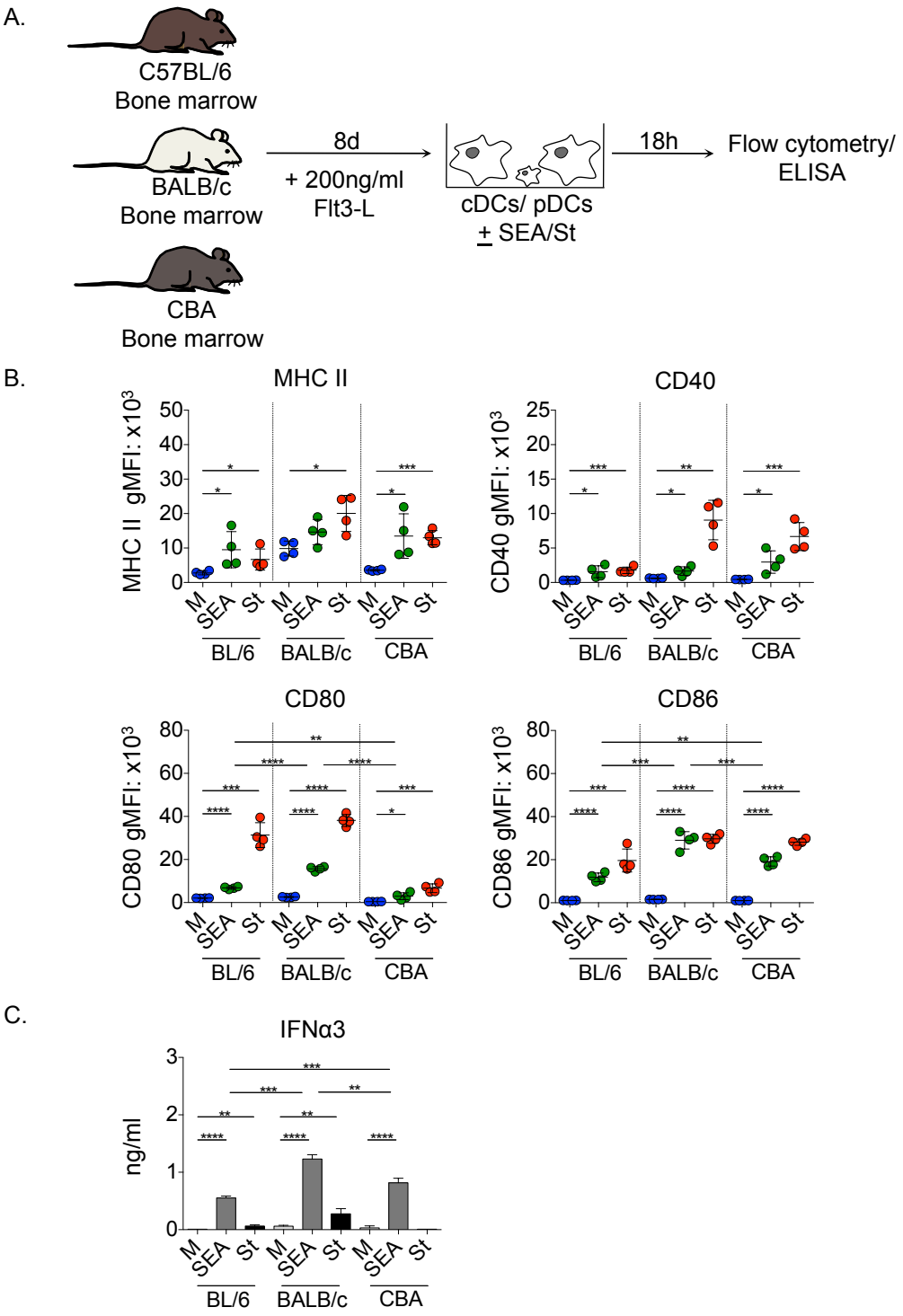


Figure 3.5 FLDCs from different mouse strains show comparable responses to SEA.
FLDCs were generated using BM from C57BL/6, BALB/c or CBA mouse strains, cells were stimulated overnight with Ag on d8 of culture and their surface phenotype analysed by flow cytometry, gMFI of activation markers was calculated (B). ELISA was performed on cell supernatants to measure levels of IFNα3 (C). Statistical difference between strains following exposure to SEA only are shown, where they exist. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data from one experiment, 4 replicate wells.

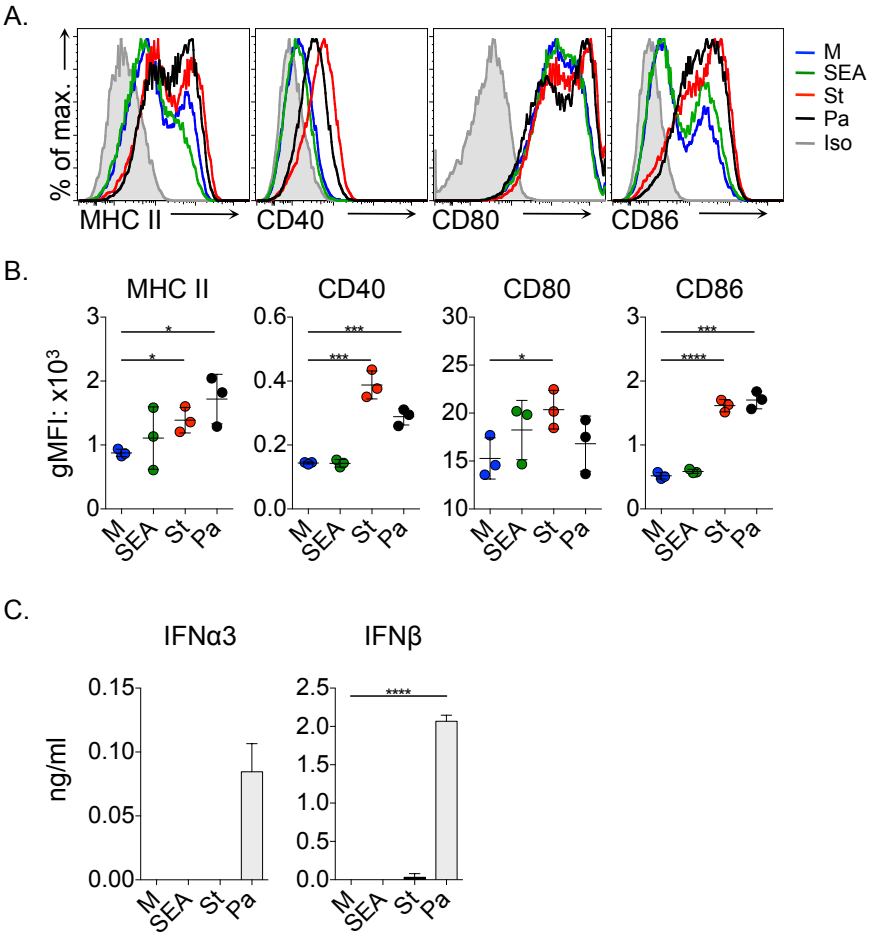


Figure 3.6 Unlike FLDCs, GMDCs fail to display clear activation in response to SEA.
Cells were cultured overnight in the presence or absence of SEA (25µg/ml), St (5µg/ml) or Pa (10µg/ml), cells were stained and analysed for their surface expression of MHC II and co-stimulatory molecules (A), gMFI for these markers was also recorded (B). IFN-I levels were ascertained by ELISA (C). Data representative of 3 (C) and 5 (A-C) experiments. *P<0.05, ***P<0.001, ****P<0.0001.

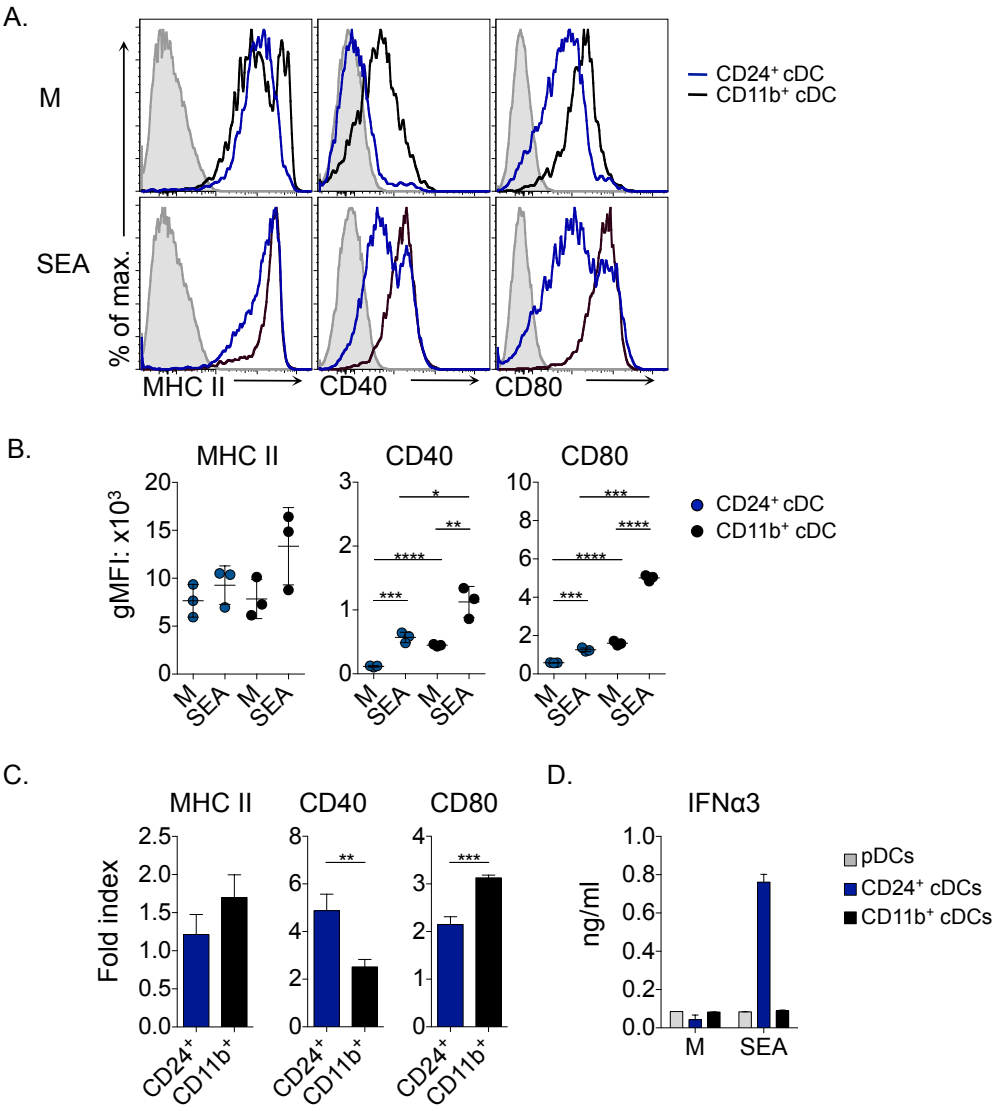


Figure 3.7 Differential response of FL-cDC subsets to SEA.
FLDCs were cultured overnight with or without SEA and their level of phenotypic activation elucidated by flow cytometry (A), gMFI for activation markers was recorded (B), and fold change in gMFI compared to basal levels was calculated for SEA stimulated cells (C). FLDC subsets were FACS sorted on d8 of culture before being replated and stimulated overnight with Ag, IFNα levels in cell supernatants was measured by ELISA (D). Data representative of 3 (A-C) and 2 (D) experiments, done in triplicate (A-C) and 4 wells (D). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

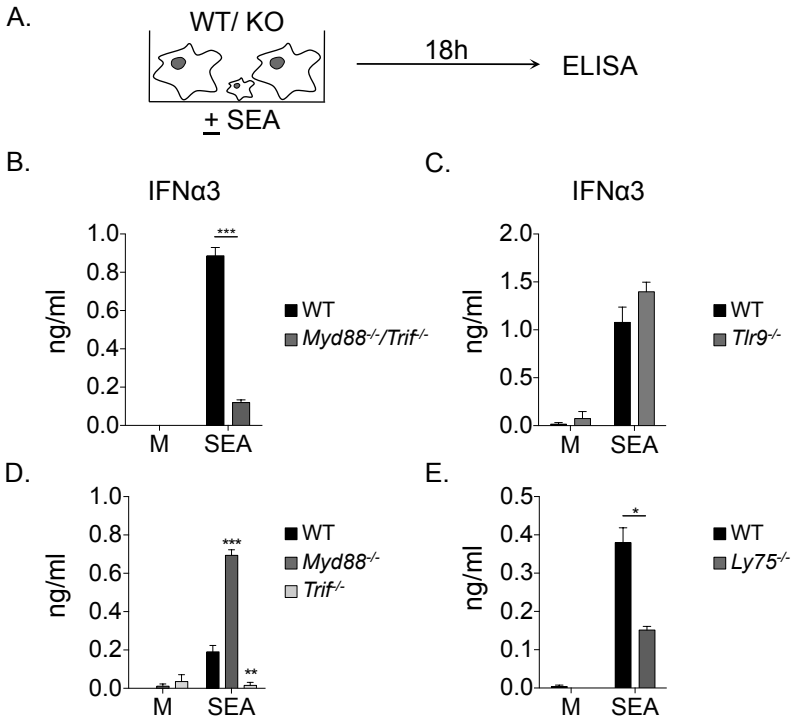


Figure 3.8 FLDC IFN-I production in response to SEA is regulated by MyD88, and promoted by TRIF and CD205 signals.
WT, *Myd88*$^{-/-}$*/Trif*$^{-/-}$ (B), *Tlr9*$^{-/-}$ (C), *Myd88*$^{-/-}$, *Trif*$^{-/-}$ (D) or *Ly75*$^{-/-}$ (CD205-deficient, E) FLDCs were cultured overnight in the presence or absence of SEA, cell supernatants were collected and analysed for IFNα secretion by ELISA. Data representative of 5 (B), 4 (C-D) and 3 (E) experiments. *P<0.05, **P<0.01, ***P<0.001. Significance levels in D. denote the difference between WT SEA and KO levels in both cases.

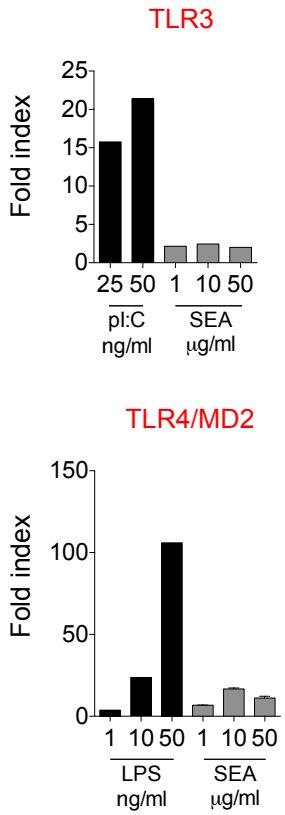
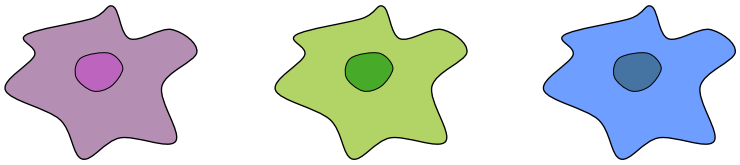


Figure 3.9 IL-8 production by TLR3 or TLR4-expressing cell lines following Ag stimulation
HEK cells stably expressing either TLR3 or TLR4/MD2 were cultured with or without Ag. Cell supernatants were collected after 22h and ELISA performed to establish IL-8 production. Data represented as fold induction of IL-8 compared to medium alone. Representative of single wells for SEA TLR3, pI:C TLR3 and LPS TLR4, duplicate wells for SEA TLR4.

Flt3-L dependent



+ SEA	$CD24^+$ cDC	$CD11b^+$ cDC	pDC
MHC II	↑	↑	~
CD40	↑↑	↑	↑
CD80	↑	↑↑	~
CD86	↑↑	↑↑	↑
Cytokine	IFN-I	~	~

GM-CSF dependent

+ SEA	GMDC
MHC II	~
CD40	~
CD80	~
CD86	~
Cytokine	~

+ St	All BM cDCs
MHC II	↑
CD40	↑↑↑
CD80	↑↑↑
CD86	↑↑
Cytokine	IL-12, IL-6, IL-10, (IFN-I), TNFα

Figure 3.10 BMDC responses to SEA and St
Changes in BMDC phenotype following overnight exposure to SEA or St. It should be noted that there is some low-level IL-12p40 and IL-6 production from FLDCs exposed to SEA, which most likely is produced by cDC subsets.

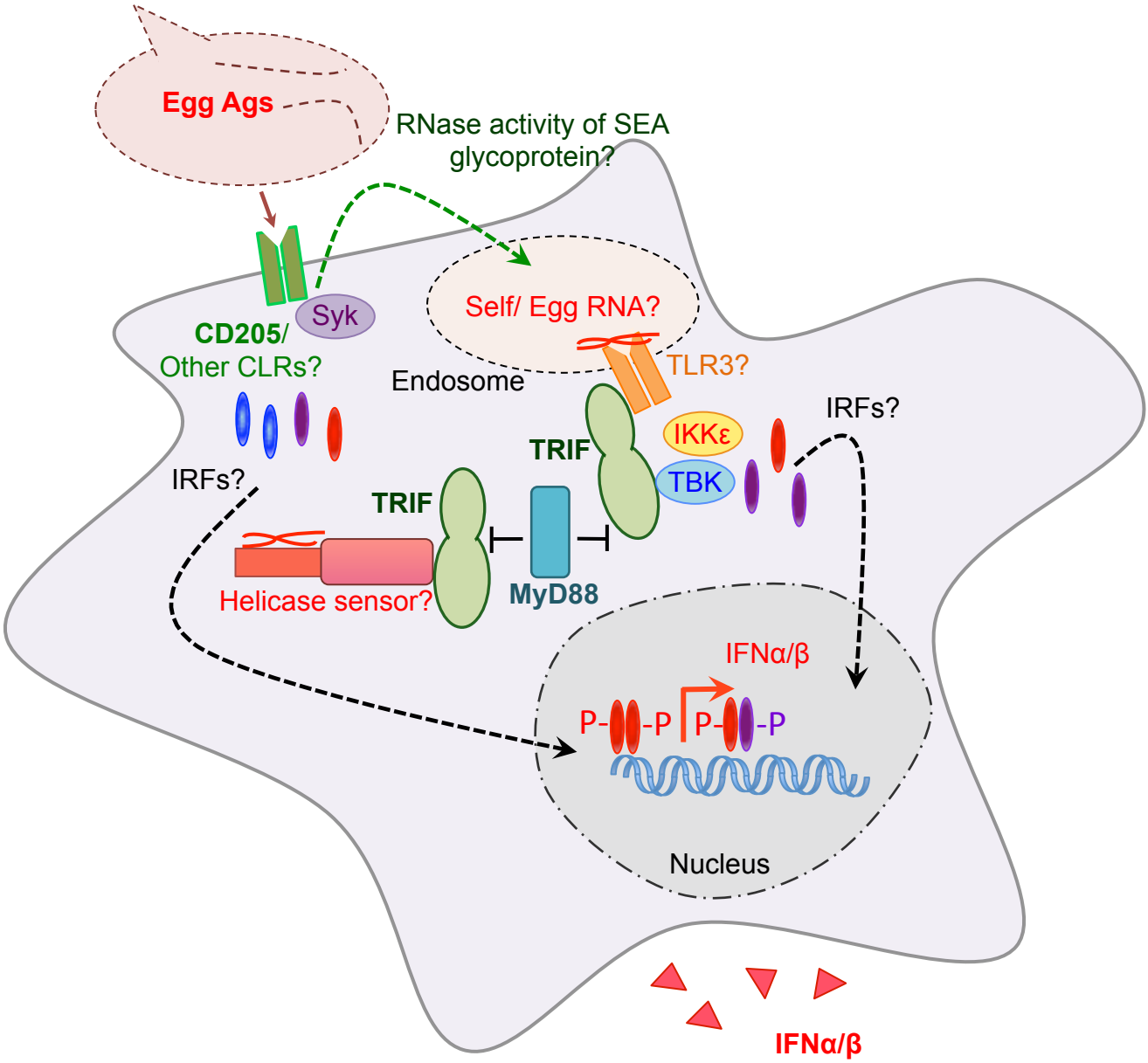


Figure 3.11 Potential PRR pathways in SEA sensing and IFN-I induction
TRIF is an essential component in the activation of the IFN-I response against SEA in FLDCs. As yet, we have not identified which PRR signals via TRIF to stimulate this response. Candidates include TLR3 and a cytosolic nucleic acid sensor, both of these receptors recognise nucleic acid ligands. Optimal IFN-I production in response to SEA also requires the CLR CD205. It is possible that CD205 can directly activate IFN-I transcription, as has been demonstrated downstream of dectin-1. Alternatively, CD205 may facilitate the internalisation of a bioactive SEA component, capable of generating nucleic acid ligands for other PRRs. It is likely that the kinases TBK and/ or IKKε, and IRFs, are involved in the signalling cascade that leads to the activation of IFN-I genes. MyD88 negatively regulates the IFN-I response, most likely by the modulation of TRIF or its downstream signalling pathways.

4.0 FLDC ACTIVATION OF TH2 RESPONSES – A ROLE FOR TYPE I IFN

4.1 Introduction

The primary role of DCs is to present Ag to naïve T cells in the secondary lymphoid organs (SLO) in order to initiate adaptive immune responses (Alvarez *et al.*, 2008; Kapsenberg, 2003). In the tissues, DCs act as sentinels, collecting antigenic material before trafficking, via the lymphatic system, to the SLO to interact with lymphocytes. Thus, efficient and accurate migration is key to DC function. cDC migration to the LN is a complex, multi-step process, requiring detachment from structural elements within the tissues, followed by entry to, and then transit in, the afferent lymphatics (Alvarez *et al.*, 2008). Trafficking of cDCs via the lymphatics is thought to be dependent on their expression of the chemokine receptor CCR7 (Tal *et al.*, 2011), under inflammatory conditions and in the steady state (Ohl *et al.*, 2004). Given that CCR7 is important for cDC migration during inflammation and in the steady state, it is likely that this receptor plays a role in DC migration in Th2 settings, however, the chemokine receptor-ligand requirements for cDC migration to the dLN following exposure to Th2-polarising Ags has not been addressed. In contrast to conventional DC subsets, pDCs are thought to primarily gain access to LN via the blood system, but only under inflammatory conditions (Diacovo *et al.*, 2005).

A published study indicated that FLDCs could migrate effectively to the dLN following subcutaneous injection (Xu *et al.*, 2007). However, the authors did not address whether both FL-cDCs and FL-pDCs reached the dLN via this route. It was also not shown exactly where FLDCs were localised within the dLNs. This is a particularly pertinent question in the context of Th2 induction in response to helminths, as a recent study suggested that during infection with the gastrointestinal parasite *H. polygyrus*, DC:T cell interactions occur outside of the T cell zone (Leon *et al.*, 2012), contrary to what may have been expected.

In this chapter, we have used an adoptive transfer model to address these issues in the context of SEA stimulated FLDCs, with confocal microscopy providing an ideal technique to identify DC localisation within the dLN. Xu *et al*. (2007) did not demonstrate that FLDCs could prime adaptive responses following injection and, up to now, there have not been any published studies to address whether FLDCs can polarise Th2 responses against

helminth Ag. Thus, this chapter also focuses on the ability of FLDCs to prime SEA-specific T cell responses *in vivo*.

In vitro studies indicate that FL-cDCs can effectively present OVA to OTII TCR Tg $CD4^+$ T cells, resulting in T cell proliferation and cytokine production, to a comparable level to that induced by GMDC or purified splenic subsets (Brasel *et al.*, 2000; Naik *et al.*, 2005; Xu *et al.*, 2007). Unlike FLDCs, SEA-specific Th2 induction by GMDCs has been studied extensively (MacDonald and Maizels, 2008). Experiments detailed in this chapter aim to redress the balance, investigating the surface markers and signalling molecules required for Th2 induction by FLDCs *in vivo*.

In chapter 3, an SEA-specific IFN-I response from FLDCs was described. Several studies have suggested that expression of the IFN-I receptor, IFNAR, is required for optimal DC function (Kurche *et al.*, 2012; Mattei *et al.*, 2009; Montoya *et al.*, 2002). However, these studies have focused wholly on the importance of IFNAR for DC function in non-Th2 settings. Thus, the final part of this chapter examines the importance of IFN-I responsiveness for effective Th2 induction by FLDCs, using both *in vitro* and *in vivo* models.

4.2 Results

4.2.1 FLDC Migration *in vivo*

An assay commonly used in our lab to assess the T cell priming capacity of BMDCs involves adoptive transfer of Ag-pulsed cells into naïve recipients (Cook *et al.*, 2012; MacDonald *et al.*, 2002c; Perona-Wright *et al.*, 2012). The response generated by transferred cells is evaluated by quantifying the T cell cytokine output from Ag-restimulated dLN cells. The requirement for GMDC MHC II expression (MacDonald *et al.*, 2001) strongly indicates that transferred DCs migrate to the draining LNs to interact with responding T cells, though this has never formally been experimentally addressed.

Before investigating the priming ability of FLDCs, we first addressed whether they were able to migrate effectively to the dLN following transfer. For this experiment, FLDCs were generated from the BM of dsRed mice; these mice express red fluorescent protein under control of the actin promoter, thus all cells fluoresce red. dsRed DCs were injected into each hind foot and the dLNs were harvested at 24 or 48h after injection. These timepoints were assessed because most surviving cells should reach the LN within the first few days (Tal *et al.*, 2011; Xu *et al.*, 2007). Prior to transfer, cells were activated overnight with SEA or St, or cultured in medium alone. 24h after injection, $CD11c^+dsRed^+$ cells were detectable by flow cytometry in the dLNs of recipient mice, and this population was present regardless of prior stimulation (Fig. 4.1B). $CD11c^+dsRed^+$ cells were also detectable in the dLNs of recipients in all groups at 48h (Fig. 4.1B) post-injection. These findings demonstrate that FLDCs are capable of efficient migration to the draining LNs of recipient mice following adoptive transfer. No consistent differences in the kinetics of migration between the three conditions were found across independent experiments.

At each timepoint, dLNs were also collected and prepared for analysis by confocal microscopy. Fig. 4.1C depicts representative images from 48h after injection. $DsRed^+$ cells were detected in dLN sections from all groups at this timepoint, and were mainly localised in areas where $TCR\beta^+$ $CD4^+$ cells were abundant (Fig. 4.1C). At higher magnification it was also possible to see dsRed cells in close association with $CD4^+$ cells (Fig. 4.1C, right panel). These data illustrate that transferred cells localised to the T cell zone of the recipient dLNs, and suggest that DCs likely interact with T cells within that area.

At all timepoints analysed, we were unable to detect any $CD45R^+$ ($B220^+$) $dsRed^+$ cells in dLN sections from mice in any groups (Fig. 4.1C). Similarly, we did not see any $CD45R^+$ $CD11c^+$ $dsRed^+$ cells by flow cytometry. Failure to detect any $CD45R^+$ transferred cells by either of these techniques suggests that pDCs did not reach the popliteal LNs following subcutaneous injection. pDCs are not thought to traffic via the lymphatics, instead entering lymphoid tissues from the blood via HEV, in a CCR7-independent manner (Diacovo *et al.*, 2005). In accordance with this, FL-pDCs expressed only negligible levels of CCR7, regardless of whether they were stimulated with Ag or not (Fig. 4.2A-C). In stark contrast, upwards of 95% of $CD24^+$ cDCs (the $CD8\alpha^+$ equivalents) and $CD11b^+$ cDCs expressed CCR7 in response to overnight culture in the presence of St (Fig. 4.2A-B). This was a significant increase compared to cells cultured in medium alone, where less than 20% of $CD11b^+$ cDCs and around 5% of $CD24^+$ cells were $CCR7^+$ in the absence of Ag. Following 18h exposure to St, cDC subsets also had significantly higher levels of CCR7 on their surface than cells in medium alone, measured by gMFI (Fig. 4.2B). The mean gMFI of CCR7 on St-stimulated $CD24^+$ cDCs was 4810, marginally higher than on $CD11b^+$ cells whose mean gMFI for this marker was 2880, representing a significant ($p=0.0098$) difference in CCR7 expression between these subsets.

Exposure of FLDCs to SEA did not induce CCR7 expression to the level that was detected in response to St (Fig. 4.2A-C; expression levels were approximately 55- and 15-fold lower on $CD24^+$ and $CD11b^+$ cDCs, respectively). Less than half of $CD11b^+$ cDCs were $CCR7^+$ after SEA exposure, and an even smaller proportion of $CD24^+$ cells expressed this chemokine receptor in response to SEA (Fig. 4.2A-B). Nevertheless, both subsets did display a significant increase in percentage positive and gMFI for CCR7, compared to cells cultured in medium alone.

The migration of pDCs via HEVs requires their expression of CCR5 (Diacovo *et al.*, 2005), thus we investigated the expression level of this receptor on the surface of FL-pDCs (Fig. 4.2D). pDCs did not express this receptor at high levels and the proportion of $CCR5^+$ cells actually went down in response to Ag stimulation. The cDC subsets also expressed only very low levels of CCR5 and expression levels were unaltered by the presence of Ag (data not shown).

4.2.2 FLDCs can effectively prime Ag-specific T cell responses

Having established that FL-cDCs were able to traffic efficiently to the dLN following adoptive transfer, we then assessed their ability to prime Ag-specific T cell responses *in vivo*. Cells were cultured with or without Ag overnight then adoptively transferred as before. We transferred the mixed DC culture generated by Flt3-L rather than pure populations of the different subsets, so as to avoid the negative impact that FACS sorting had on the cells. To purify enough cells for transfer would have required a prohibitively large number of cells at the outset, as well as an extended length of time on the FACS sorter, likely leading to a further reduction in cell viability. Moreover, the migration experiments indicated that pDCs did not migrate to the pLNs and so were unlikely to contribute in this model. 7d after injection, dLN were harvested and cells restimulated with Ag *in vitro*. It was clear that FL-DCs cultured in the presence of SEA prior to transfer successfully primed Ag-specific Th2 response as high levels of IL-4, IL-5, IL-10 and IL-13 were measured in supernatants from restimulated dLN cells (Fig. 4.3B). Additionally, FLDCs exposed to SEA prior to transfer induced IL-17 and IFNγ production, although, unlike the Th2 cytokines, secretion of these was not significantly above background levels (Fig. 4.3B). FLDCs cultured with St effectively generated an Ag-specific Th1/Th17 response following transfer, characterised by significant levels of IL-17 and IFNγ production upon restimulation of dLN cells, whilst Th2 cytokines were undetectable (Fig. 4.3C). The St-specific response also included a regulatory aspect, as IL-10 was elevated in the supernatants of restimulated LN cells (Fig. 4.3C). Together, these findings illustrate that FL-cDCs successfully generated Ag-specific T cell responses in the dLN following adoptive transfer.

4.2.3 FLDC priming of SEA-specific Th2 responses requires MHC II and CD40 expression

Previous *in vivo* experiments with GMDCs have established that SEA-specific Th2 induction by DCs is dependent on their expression of MHC II, CD40 (MacDonald *et al.*, 2001; MacDonald *et al.*, 2002c), whilst DC-derived IL-10 has no role in this process (Perona-Wright *et al.*, 2006a). Although it was clear that FL-cDCs were able to prime Th2 responses, our findings from chapter 3 illustrated that FL-cDCs display a distinct phenotype from GMDCs when exposed to SEA. For this reason, we undertook to establish whether the published findings for GMDCs were valid for FLDCs.

Firstly, transfer of FLDC derived from *H2-ab*$^{-/-}$ mice (*Ab*$^{-/-}$, lacking a subunit of MHC II) into WT recipients confirmed that SEA-specific Th2 induction by FLDCs was dependent on their expression of MHC II, as *H2-ab*$^{-/-}$ cells failed to prime levels of IL-4, 5 and 13 significantly above background (Fig. 4.4B). No IL-10 was detected from restimulated pLN cells in any group in this experiment. *Cd40*$^{-/-}$ FLDCs displayed an impaired ability to generate SEA-specific Th2 responses, though the reduction in IL-5 and IL-13 compared to WT did not reach significance due to variability in the WT cytokine levels (Fig. 4.4C). Finally, FLDC induction of SEA-specific Th2 responses did not require DC-derived IL-10 as Th2 cytokine levels generated by WT and *Il10*$^{-/-}$ DCs were comparable (Fig. 4.5). Taken together, these experiments reveal that Th2 induction by FLDCs, in common with GMDCs, is dependent on their expression of MHC II and CD40, but that DC-derived IL-10 has no major role in this process.

4.2.4 A central role for IFN-I in Th2 induction by FLDCs

In chapter 3, it was shown that CD24^{+} cDCs secrete IFN-I in response to SEA exposure. A number of studies have suggested a role for IFN-I in maintaining DC function, often in an autocrine manner (Montoya *et al.*, 2002). We wanted to investigate whether the SEA-specific IFN-I signal was required for optimal FLDC function. As discussed previously, the expansive nature of the IFN-I family means it is not feasible to knock out or block IFN-I production, so instead we addressed the more general question - if FLDCs were unable to respond to any IFN-I signal, could they still prime effectively in a Th2 setting? To do this, WT or IFNAR-deficient FLDCs were cultured overnight with SEA and adoptively transferred into WT recipients. Notably, *Ifnar1*$^{-/-}$ FLDCs displayed a severe impairment in their ability to prime SEA-specific IL-4, IL-5, IL-10 and IL-13 (Fig. 4.6), with the levels of all Th2 cytokines remaining at baseline levels. Production of SEA-specific IFNγ was not significantly affected, whilst only very low levels of IL-17 were detected. This result indicated that IFN-I receptor expression by FLDCs is critical for their ability to generate SEA-specific Th2 responses *in vivo*.

4.2.5 IFNAR-deficient FLDCs display defective responses to SEA stimulation

In order to investigate why *Ifnar1*$^{-/-}$ FLDCs were unable to prime *in vivo* Th2 differentiation we first assessed their *in vitro* response to SEA treatment. SEA exposure did not induce the intermediate activation phenotype characteristic of WT cells in *Ifnar1*$^{-/-}$ FLDCs, with

cDCs showing reduced MHC II expression compared to WT cells (Fig. 4.7A). Strikingly, the gMFI of CD40 and CD86 on *Ifnar1*$^{-/-}$ FL-cDCs following SEA exposure was barely above background levels and was significantly lower than on WT cells cultured with SEA (Fig. 4.7A). Surface phenotype was not the only aspect of the *Ifnar1*$^{-/-}$ FLDC response that was affected, as receptor-deficient cells also secreted significantly less IFNα3 in response to SEA than WT cells (Fig. 4.7B).

Signalling downstream of IFNAR results in sustained production of IFN-I (Malmgaard *et al.*, 2002; Marie *et al.*, 1998). Similarly, there are a large number of other interferon-stimulated genes (ISG) whose transcription is dependent on signalling via IFNAR. We reasoned that the SEA-induced IFN-I would act in an autocrine manner, and so assessed the expression levels of other ISGs by FLDCs after a short (6h) culture with SEA. We found that SEA stimulated a significant increase in the expression of two well-known ISGs – Ifit1 and Mx1 in WT FLDCs (Fig. 4.7C). SEA-induced upregulation of these genes was not evident in *Ifnar1*$^{-/-}$ FLDCs. In addition, baseline expression of these ISGs by unstimulated medium control *Ifnar1*$^{-/-}$ cells was reduced compared to WT controls (Fig. 4.7C). Thus, in the absence of functional IFNAR, not only were FLDC phenotypic responses to SEA abrogated, but the induction of the IFN-I signature was also curtailed.

4.2.6 IFNAR-deficient FLDCs develop normally with only minor differences in subset composition

To address whether the reduced ability of *Ifnar1*$^{-/-}$ FLDCs to be activated by SEA and prime Th2 responses was due to impaired DC generation or viability we compared the viability of WT and IFNAR-deficient FLDCs. Unstimulated *Ifnar1*$^{-/-}$ FLDCs had comparable viability to WT cells, with a similar percentage of live cells and equivalent proportions of cells undergoing apoptosis (Fig. 4.8B). In the experiment depicted here there was a small but significant increase in viable IFNAR-deficient cells after overnight culture with SEA. However this was subject to variability across experiments. It should be highlighted that FLDCs have quite a high level of apoptosis, which has not previously been reported (Fig. 4.8B).

The percentage of live-singlet cells that expressed CD11c was comparable between WT and *Ifnar1*$^{-/-}$ FLDCs (Fig. 4.8C). Within the CD11c^{+} population, *Ifnar1*$^{-/-}$ Flt3-L cultures had

significantly less CD45R^{hi} pDCs than WT and a consequent increase in the proportion of CD45R^{lo} cDCs (Fig. 4.9A). There were also changes in the composition of the CD45R^{lo} cDC compartment in *Ifnar1*$^{-/-}$ cultures, with approximately double the proportion of the CD24^{+} cDCs, whilst the percentage of CD11b^{+} cDCs dropped by one fifth (Fig. 4.9B). It is clear that Ifnar1 is not required for generation of FLDCs. However the lack of functional receptor expression on BM precursors did have some effect on the populations generated by Flt3-L.

4.2.7 In the absence of TLR signalling FLDCs cannot prime Th2 responses

Having established that *Ifnar1*$^{-/-}$ FLDCs displayed no major impairment in development or survival, but showed some differences to WT FLDCs, we attempted to establish whether an inability to secrete IFN-I was the key factor in this response. As shown in chapter 3, *Myd88*$^{-/-}$*/Trif*$^{-/-}$ FLDCs also fail to secrete IFN-I (Fig. 3.8A). For this reason, we assessed whether *Myd88*$^{-/-}$*/Trif*$^{-/-}$ FLDCs showed a similar deficiency in SEA-driven Th2 induction *in vivo*. This revealed that Th2 induction, as well as IFNγ (no IL-17 was detected), was completely ablated in the absence of the TLR adaptor proteins (Fig. 4.10B). This result may suggest that IFN-I production by FLDCs following exposure to SEA is essential for their ability to prime Th2 responses, however, it cannot be ruled out that other defects in *Myd88*$^{-/-}$*/Trif*$^{-/-}$ FLDC function are responsible for their inability to prime SEA-specific Th2 responses.

4.2.8 IFNAR-deficient FLDCs prime normal T cell activation *in vitro*

To directly address whether *Ifnar1*$^{-/-}$ FLDCs had a specific defect in their ability to interact with T cells, we used a more reductionist *in vitro* model of T cell priming. In the absence of an SEA-specific Tg T cell model, it was necessary to use the OVA system to investigate the activation of naïve CD4 cells (Barnden *et al.*, 1998; Murphy *et al.*, 1990). First, we used OTII TCR Tg CD4^{+} T cells to assess the ability of FLDCs to induce Ag-specific T cell proliferation, as this assay provides a good measure of Ag uptake, processing and presentation. FLDCs were FACS sorted on day 8 of culture to provide a pure cDC population. We excluded pDCs on the basis that they do not appear to play a role in the *in vivo* transfer model. Sorted cDCs were cultured with CFSE-labelled OTII TCR Tg T cells in the presence or absence of OVA peptide (pOVA) or whole OVA protein (OVA). Both WT and *Ifnar1*$^{-/-}$ FL-cDCs successfully stimulated T cell proliferation in the presence of OVA

peptide, with almost 100% of T cells having undergone at least one round of division after 96h of culture (Fig. 4.11B). Similarly, all but a very small percentage of T cells proliferated when OVA protein was present, regardless of whether the presenting DCs were WT or *Ifnar1*$^{-/-}$ (Fig. 4.11C). Since whole OVA protein must be processed to generate peptides to be presented on MHC II, this indicates that *Ifnar1*$^{-/-}$ FL-cDCs are not inherently defective in Ag processing and presentation.

We next addressed whether *Ifnar1*$^{-/-}$ FL-cDCs had a specific deficiency in their ability to polarise Th2 responses *in vitro*. As OTII TCR Tg T cells produced little to no T cell cytokines following culture with OVA or pOVA, to specifically assess Th2 priming we activated a polyclonal T cell population with anti-CD3 in the presence of IL-4 (Cook *et al.*, 2012). Although IL-4 acts as a Th2-polarising agent in these cultures, DCs are also essential, and there is no Th2 induction in their absence. Polyclonal CD4^{+} T cells were FACS sorted from KN2xIL-13eGFP (Fig. 4.12) or KN2xIL-10eGFP (Fig. 4.13) reporter mice. Cells from these mice express human CD2 (huCD2) on their surface when IL-4 is secreted (Mohrs *et al.*, 2005), and produce GFP when IL-13 (IL-13eGFP mice) or IL-10 (IL-10eGFP mice) message is transcribed. In the absence of exogenous IL-4, very few T cells expressed huCD2 (Fig4.12B) or IL-13eGFP (Fig. 4.12C). With the addition of IL-4 there was a significant increase in the percentage of huCD2^{+} and IL-13eGFP^{+} cells, and this was the case in the presence of either WT or *Ifnar1*$^{-/-}$ cDCs (Fig. 4.12B-C). Similarly, IL-10eGFP expression was induced by exogenous IL-4 with either WT or IFNAR-deficient cDCs (Fig. 4.13). This assay demonstrates that IFNAR-deficient FL-cDCs can effectively facilitate T2 polarisation in the presence of IL-4.

4.2.9 A migration defect in IFNAR-deficient FL-cDCs?

We had generated a growing body of evidence that *Ifnar1*$^{-/-}$ cDCs could still function effectively as APCs *in vitro*, despite defective responses to SEA and an inability to prime T cell cytokine production *in vivo*. As well as eliciting upregulation of MHC II and co-stimulatory molecules, SEA also caused significant increase in CCR7 expression on a proportion of FL-cDCs, primarily CD11b^{+} cDCs (Fig. 4.2A-B and Fig. 4.14, WT data for both figures are from the same experiment). As with other surface markers, SEA-specific CCR7 expression on *Ifnar1*$^{-/-}$ cDCs was significantly reduced compared to WT cells (Fig. 4.14). This was particularly apparent with CD11b^{+} cDCs, as both the percentage of CCR7^{+}

cells, and the CCR7 gMFI, were at baseline levels on *Ifnar1*$^{-/-}$ cells exposed to SEA (Fg4.14B-C). This suggests that functional IFN-I signalling may be required for effective FLcDC migration to the draining LN following adoptive transfer.

To compare migration of WT and *Ifnar1*$^{-/-}$ DCs we decided to first assess their chemotactic activity in an *in vitro* transwell assay of chemotaxis. DCs were cultured overnight with or without SEA, and then washed and resuspended in serum-free medium. This was done because components of FCS, which is normally present in FLDC cultures, can activate chemokinesis and block responsiveness to chemokines. Cells were placed in the top chamber of transwells, with the bottom chamber containing varying concentrations of the CCR7 ligand, CCL21 (Fig. 4.15A). We expected that CCR7-expressing cells in the upper section would be attracted by CCL21 and traverse the permeable membrane into the lower chamber. After 3h incubation cells present in the lower chamber were stained and analysed by a MACSQuant flow cytometer that can provide accurate cell counts. These were used to calculate the chemotactic index of the various DC subsets from the different conditions (Fig. 4.15A).

In the presence of CCL21, the number of WT CD24^{+} and CD11b^{+} cDCs that traversed the membrane increased by approximately 4-fold compared to medium alone (Fig. 4.15B). However, there was no further dose-dependent increase in migration with increasing concentrations of CCL21 (Fig. 4.15B). Only unstimulated CD11b^{+} *Ifnar1*$^{-/-}$ cDCs displayed a significant defect in transmigration compared to WT, with a chemotactic index that was two-fold lower in the presence of CCL21 than WT cells (Fig. 4.15B). Prior exposure to SEA seemed to reduce the number of WT CD24^{+} cDCs that transmigrated. However, the chemotactic index of *Ifnar1*$^{-/-}$ CD24^{+} cDCs, and both WT and IFNAR-deficient CD11b^{+} cDCs was unaffected by SEA.

The unreliability of this transwell assay as a measure of migratory capacity was highlighted by the fact that pDCs were detectable in all conditions (Fig. 4.16A), despite only 0.5-1.5% of pDCs expressing CCR7 (Fig. 4.16B). This suggests that the presence of pDCs in the bottom chamber is due to random movement and not chemotaxis. This finding indicated that another method of measuring DC migration was required to analyse the role of IFNAR in this process.

We had previously found that adoptive transfer of dsRed$^+$ cells provided an *in vivo* assay for DC migration (Fig. 4.1). In the absence of an *Ifnar1*$^{-/-}$ x dsRed cross, we attempted this assay using CD45.2$^+$ FLDCs transferred into CD45.1$^+$ naïve recipients. Initially, by analysing the size of the CD45.2$^+$ CD11c$^+$ population present in the dLN by flow cytometry we thought that this could provide a good measure of migration. However, injection of cells caused infiltration of a CD45.1$^+$ CD45.2$^+$ population that could be found in the CD45.2$^+$ CD11c$^+$ gate of injected animals (Fig. 4.17B-D). Once this autofluorescent population was excluded, it was possible to identify a WT CD45.1$^-$ CD45.2$^+$ CD11c$^+$ population in the dLN of some recipient mice, for example animal 1 (Fig. 4.17C), but not others (animal 2, Fig. 4.17D), suggesting that not all injections had resulted in successful migration. In addition, no CD45.2$^+$ cells were detectable in dLN sections analysed by confocal microscopy.

4.2.10 GMDCs do not require Ifnar1 expression to effectively induce Th2 responses

Although GMDCs did not secrete IFN-I in response to SEA stimulation (Fig. 3.6C), a published study suggested that they too require IFN-I responsiveness to be able to stimulate optimal OVA-specific T cell proliferation *in vitro* (Montoya *et al.*, 2002). However, the authors did not address whether Ag-specific cytokine output from T cells was affected in the presence of IFNAR-deficient GMDCs. We found that WT and *Ifnar1*$^{-/-}$ GMDCs generated comparable SEA-specific Th2 cytokine responses in the dLN of recipient mice (Fig. 4.18A). In fact, IL-4 levels elicited by IFNAR-deficient cells were significantly increased compared to WT (Fig. 4.18A). Similarly to IFNAR-deficient FL-cDCs, *Ifnar1*$^{-/-}$ GMDCs had no defect in their ability to stimulate Ag-specific proliferation from OTII TCR Tg T cells in the presence of OVA protein or peptide (Fig. 4.18B), or to generate a Th2 profile from reporter T cells in the IL-4-dependent *in vitro* assay described in section 4.2.8 (Fig. 4.18C-D). Induction of IL-10eGFP by IFNAR-deficient GMDCs was significantly increased compared to WT cells (Fig. 4.18E). These data clearly show IFN-I is not required for GMDCs to function as APCs in a Th2 setting either *in vitro* or *in vivo*.

Despite a previous report that IFNAR-deficient splenic DCs are more resistant to apoptosis (Mattei *et al.*, 2009), as with FL-cDCs, we found no difference in the viability of *Ifnar1*$^{-/-}$ GMDCs compared to WT cells, when measured by 7-AAD and Annexin V staining (Fig. 4.18F).

Differential expression of the IFNAR may explain why FLDCs, but not GMDCs, depend on IFN-I for their function. Whereas only 20% of GMDCs expressed IFNAR1 on their surface in the presence or absence in Ag (Fig. 4.19B), some 80% of FL-cDCs were IFNAR1$^+$ in the absence of Ag stimulation (Fig. 4.19A). As professional IFN-I producing cells, almost 100% of pDCs expressed IFNAR1 in the basal state (Fig. 2.19A). Both the percentage of IFNAR1$^+$ FLDCs and the gMFI of IFNAR1 expression were significantly decreased on BMDCs in the presence of Ag (Fig. 4.19A and C). This may reflect downregulation or degradation of the receptor following stimulation, which has been reported in studies of human monocyte-derived DCs (Severa *et al.*, 2006), and in mouse and human fibroblasts (Qian *et al.*, 2011). The antibody used in our experiments was a blocking antibody, however (Sheehan *et al.*, 2006), thus reduced staining may reflect the fact that IFNα/β is bound to the receptor.

4.2.11 Summary:

- FL-cDCs migrate to the dLN following adoptive transfer (Fig. 4.1), forming productive DC:T cell interactions to polarise an SEA-specific Th2 response (Fig. 4.3B)
- FL-pDCs do not reach the pLN after transfer (Fig. 4.1)
- Th2 priming by SEA-activated FL-cDCs is dependent on MHC II and CD40 expression (Fig. 4.4)
- Functional IFNAR signalling is required for FL-cDCs to prime effectively (Fig. 4.6)
- IFNAR-deficient FL-cDCs fail to become activated (Fig. 4.7A), generate an IFN-I signature (Fig. 4.B-C) or upregulate CCR7 (Fig. 4.14) in response to SEA
- T cell priming by IFNAR-deficient FL-cDCs is not defective *in vitro* (Fig. 4.11-13)
- GMDCs do not rely on IFNAR signalling in any Th2 setting analysed (Fig. 4.18)

4.3 Discussion

In this chapter we set out to investigate the capacity of FLDCs to prime SEA-specific Th2 responses, and the possible involvement of IFN-I in this process. For the first time, we have shown that SEA activated FLDCs effectively migrated to the dLN following adoptive transfer, where they capably induce a Th2 response. Another novel finding of our studies of FLDC SEA-specific Th2 priming was that induction of this response was dependent on DC expression of IFNAR. However, FLcDC function was not curtailed *in vitro* in the absence of functional IFNAR. This suggests that IFNAR-deficient FLcDCs may have defective migration *in vivo,* identifying a role for IFN-I in migration and Th2 priming by Flt3-L dependent cDCs.

4.3.1 Migration and priming by FLDCs

FACS and microscopy analysis following transfer of dsRed cells demonstrated that FLDCs migrated efficiently, with cells from all conditions detectable in the dLN 24h after transfer. Although cDCs cultured in medium alone or in the presence of SEA expressed much lower levels of CCR7 than cells exposed to St (Fig. 4.2A-C), there was no difference in the kinetics of cell migration (Fig. 4.1B-C). This was somewhat surprising, since we expected that the high level of CCR7 expression (essentially 100%) by FLDCs following exposure to SEA would give them a migratory advantage. One possible explanation for this discrepancy is that FLDCs cultured overnight with St have reduced migratory capacity. In a transwell assay involving transmigration of D1 cells (an immortalised DC cell line from mouse spleen, generated from cells with dendritic morphology that expand in response to GM-CSF) through an endothelial cell layer, the migratory capacity of LPS-stimulated DCs peaked at 4h, but by 18h transmigration was much reduced (Granucci *et al.*, 1999). However, the findings of Granucci *et al*. (1999) are in conflict with our work that demonstrates that cells cultured in the presence of St still express high levels of CCR7 after 18h (Fig. 4.2), suggesting a capability to migrate at later timepoints. One way to test whether migratory capacity is reduced at 18h would be to culture FLDCs with Ag for a shorter period prior to transfer (4h), and compare the migration of these cells to FLDCs cultured with Ag for 18h.

Confocal microscopy analysis of recipient dLNs demonstrated that FL-pDCs did not reach the dLN when injected subcutaneously (Fig. 4.1C). It is possible that FL-pDCs

downregulated expression of CD45R following transfer, which would explain why we were unable to detect dsRed$^+$ cells that co-expressed CD45R. However, there is no evidence in the literature that pDCs alter their expression of CD45R. pDCs do not migrate via the afferent lymphatics, as cDCs do, but rather via the blood (Diacovo *et al.*, 2005). Thus, perhaps more likely, the route of injection in this experiment did not provide pDCs easy access to the blood system. It is also possible that FL-pDCs migrating via the blood system only appeared in the LN at timepoints later than was analysed here.

If transferred FL-pDCs had disseminated systemically in the circulatory system it is possible that these cells migrated to the spleen. In our experiments, the accumulation of transferred cells in the spleen was not assessed. However, as shown by Diacovo *et al.* (2005), although pDCs can attach to noninflamed HEV, an inflammatory stimulus is required for them to gain entry. For this reason, we may only be able to visualise pDCs in lymphoid organs following co-injection of an inflammatory stimulus, such as LPS or CpG. Even so, the process of pDC transmigration via HEVs requires CCR5 (Diacovo *et al.*, 2005), which was not obviously expressed on our *in vitro* differentiated FL-pDCs (either basally, or following exposure to SEA or St; Fig. 4.2D). The absence of pDCs in the dLN following adoptive transfer suggests that they are unlikely to have a role in priming T cell responses there in this model, although it is possible they may play a role at the injection site.

FACS analysis clearly showed that FL-cDCs could migrate efficiently to the dLN, with confocal microscopy confirming that the majority localised to the T cell zone (Fig. 4.1C). Contrary to the finding of Léon *et al*. (2012) that DCs localise to the edges of the B cell zone during *H. polygyrus* infection, most dsRed$^+$ cells were situated well within the T cell zone. This discrepancy may reflect the fact that our experiments analysed the localisation of transferred cells cultured with Th2-polarising Ag rather than *in vivo* DCs responding to helminth infection. Alternatively, DC localisation may be site-specific (popliteal LN vs. MLN) or pathogen/Ag-specific (SEA vs. *H. polygyrus*).

High magnification revealed that, in some cases, dsRed$^+$ DCs were very closely associated with CD4$^+$ cells, suggesting that transferred cells present Ag to T cells of the dLN. In agreement with this hypothesis, FLDCs cultured in the presence of SEA prior to

transfer induced Ag specific Th2 responses (IL-4, 5, 10 and 13), detectable seven days after transfer. Conversely, cells exposed to St prior to transfer induced significant Ag-specific IL-10, IL-17 and IFNγ (Fig. 4.3C), but no IL-4, IL-5 or IL-13 was detectable. Thus FLDCs induce appropriate polarised T cell responses to helminth and bacterial Ag following adoptive transfer *in vivo*.

FLDC bulk cultures were capable inducers of an SEA-specific Th2 response following transfer. However, what is clear when comparing the cytokine output from several experiments is that this response can be quite variable (Fig. 4.3-4.6). Flt3-L cultures are heterogeneous (Fig. 3.1) and the proportions of the different subsets they generate can vary between experiments. This may explain why IL-4 and IL-5 output ranged from 0.05ng/ml to 3ng/ml. Other reasons for experiment-to-experiment variability include differences in the age or sex of mice used, although every effort was made to avoid these variables when possible. It is also likely that there was variability in the length of time that DCs were exposed to Ag, as well as the length of time between harvesting LNs and putting LN cells into culture, similarly, the amount of time that LN cells were in culture may not be exactly 72h for each replicate experiment. However, this kind of variability in absolute cytokine levels is the norm rather than the exception in *ex vivo*/ *in vitro* systems that use primary cells and complex Ag mixes. More importantly, the patterns of cytokine production are robust and reproducible despite some variability. WT DCs exposed to SEA prior to transfer reproducibly polarise Th2 cytokine production in the dLNs, cytokines that are not induced by unstimulated DCs or cells exposed to a bacterial stimulus. DCs can induce some level of IFNγ and IL-17 production following exposure to SEA, however, this is uniquely associated with strong induction of Ag-specific Th2 cytokines.

4.3.2 The role of IFN-I in Th2 induction by FLDCs

Initial experiments revealed that FLDC Th2 induction was similar to GMDC in that it required MHCII and CD40, but one clear difference was that FLDC (but not GMDC) SEA-specific Th2 induction was dependent on IFN-I responsiveness. In our adoptive transfer model, *Ifnar1*$^{-/-}$ FLDCs displayed a complete failure to prime SEA-specific Th2 responses, whilst the Th1 facet of this response was unaffected (Fig. 4.6). Unravelling exactly why these cells were ineffective in this model proved less straightforward. Part of the explanation for this may be that IFN-I signalling was clearly essential to the ability of FL-

cDCs to generate the intermediate level of surface activation characteristic of WT cells, as CD40 and CD86 expression on *Ifnar1*$^{-/-}$ cells exposed to SEA were barely above background (Fig. 4.7A).

Similarly, SEA-specific IFN-I output was not maintained in the absence of IFNAR (Fig. 4.7B). This is likely due to the breakdown of the IFN-I feedback loop, which governs sustained IFNα/β production via IFNAR (Honda and Taniguchi, 2006a). Jak/STAT signalling downstream of IFNAR initiates transcription of hundreds of other ISGs, thus the action of this receptor controls a vast array of cellular processes besides IFN-I production (Gonzalez-Navajas *et al.*, 2012). Because IFN-I readily binds to its surface receptor soon after secretion (Piehler *et al.*, 2012), it can be very difficult to detect soluble protein in supernatants by ELISA. For this reason, transcription analysis of ISGs can provide a useful biomarker for an IFN-I signature. We have identified a number of ISGs that are upregulated by FLcDCs following 6h culture with SEA, including Ifit1 and Mx1 (Fig. 4.7C). Both of these genes were found to be upregulated in a microarray of D1 cells cultured with live schistosome eggs (Trottein *et al.*, 2004). In accordance with Trottein *et al.*, we found that expression of these genes was abolished in the absence of functional IFNAR (Fig. 4.7C). As a next step in our work, a similar microarray approach to assess changes in gene expression in SEA-stimulated FLDC subsets, and their reliance on IFNAR, would provide a more comprehensive picture of ISG expression by these cells.

Understandably, the field of study around ISGs has thus far focused primarily on their role as antiviral effectors (Diamond and Farzan, 2013; Schoggins and Rice, 2011; Schoggins *et al.*, 2011). However, the broad range of the ISG response suggests they are likely to have as yet unidentified function in DCs in a number of settings, which would be inhibited in *Ifnar1*$^{-/-}$ cells. For example, members of the IFIT family can inhibit protein translation by binding to the initiation factor eIF3 (Diamond and Farzan, 2013). As such, a study with a murine macrophage cell-line that overexpressed IFIT2 found that IL-6 and TNFα production in response to LPS stimulation was inhibited (Berchtold *et al.*, 2008). Omega-1, an RNase present in SEA, has already been shown to condition DCs for priming Th2 responses by repressing protein synthesis (Everts *et al.*, 2012), demonstrating that this process contributes to the Th2-polarising ability of this immune-dominant component of

SEA. Thus, it is possible that induction of IFITs by SEA may function to limit translation and enhance Th2 induction.

The failure of IFNAR-deficient cells to induce SEA-specific Th2 responses did not appear to be linked to any substantial developmental or survival abnormalities (Fig. 4.8 and Fig. 4.9). A small number of studies do suggest that IFN-I regulate DC differentiation and survival, with Mattei *et al*. (2009) finding that *Ifnar1*$^{-/-}$ splenic DCs were more resistant to apoptosis in short-term culture. Mattei *et al.* also found impairment in *in vitro* GMDC differentiation from *Ifnar1*$^{-/-}$ BM; in contrast we have experienced no significant impact of IFNAR deficiency on GMDC or FLDC generation.

Comparing apoptosis of WT and *Ifnar1*$^{-/-}$ cells illustrated that FLDCs are more prone to apoptosis (20-30% cDC in late apotosis; Fig. 4.8A-B) than GMDC (<5%; Fig. 4.18F), This suggests that, although Flt3-L is a key differentiation factor, DCs may require other factors, such as GM-CSF, for more long term survival. This could be assessed *in vitro* by the addition of GM-CSF to DC cultures during Ag stimulation. This difference may relate to the different STATs involved in FLDC and GMDC differentiation, with STAT3 activated by Flt3 (Laouar *et al.*, 2003), whilst GM-CSF function is primarily mediated by STAT5 (Feldman *et al.*, 1997). It has been shown that GM-CSF-activated STAT5 can inhibit STAT3-mediated apoptosis induced by IL-21 in GMDCs (Wan *et al.*, 2013). GM-CSF activation of STAT5 has also been shown to protect neural cells from apoptosis (Choi *et al.*, 2011a).

As *Myd88*$^{-/-}$*/Trif*$^{-/-}$ FLDC secretion of IFNα was limited following SEA stimulation (Fig. 3.8B), we assessed the ability of these cells to prime *in vivo* Th2 responses, and found a similar complete ablation of SEA-specific T cell responses to what we had seen with *Ifnar1*$^{-/-}$ FLDCs (Fig. 4.10B). This raises some interesting questions, as Th2 priming by both SEA and during *S. mansoni* infection is currently thought to be independent of MyD88 signalling (Pearce and MacDonald, 2002; Perona-Wright *et al.*, 2006b). This conclusion was based on studies with MyD88-deficient animals for *in vitro* DC-Th2 polarisation assays, using sorted splenic cDCs, in the presence of SEA (Jankovic *et al.*, 2004), as well during patent *S.mansoni* infection (Layland *et al.*, 2005). *Tlr3*$^{-/-}$ mice infected with *S. mansoni* displayed no significant phenotype on d49 of infection (Vanhoutte *et al.*, 2008), suggesting that TRIF is also not required for immune activation in this setting. However our results with double

deficient *Myd88*$^{-/-}$*/Trif*$^{-/-}$ cells clearly show that these signals are required for schistosome-induced Th2 induction and caution against drawing firm conclusions using only singly deficient mice. Based on this, it is important that the role of MyD88 and TRIF together, as well as separately, is assessed in *S. mansoni* infection. The ability of *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ FLDCs to stimulate SEA-specific Th2 responses following adoptive transfer must also be addressed.

Whilst Montoya *et al*. (2002) reported a reduction in the ability of IFNAR-deficient GMDCs to stimulate naïve T cell proliferation *in vitro*, we did not see this for either FL-cDCs (Fig. 4.11) or GMDCs (Fig. 4.18B). In fact, *Ifnar1*$^{-/-}$ FL-cDCs could stimulate CD4^{+} T cell proliferation in response to whole OVA (Fig. 4.11C), indicating they have no major defect in Ag uptake, processing and presentation. Similarly, *Ifnar1*$^{-/-}$ cells were also proficient in generating hallmarks of a Th2 response from polyclonal T cells in an IL-4-dependent *in vitro* model (Fig. 4.12-13). Whilst this assay demonstrates that IFNAR-deficient FL-cDCs can facilitate Th2 polarisation driven by IL-4, this assay does not definitively show that these cells are capable of direct Th2 priming. In order to demonstrate this effectively, we require transgenic T cells with a TCR specific for a component of SEA, as SEA-specific Th2 polarisation cannot be detected from a polyclonal population of T cells *in vitro*. Using cytokine reporter mice to measure Th2 polarisation by SEA-exposed BMDCs following adoptive transfer has also so far proved unsuccessful (Peter Cook, unpublished data), however, it may be possible to optimise this technique to more directly measure Th2 polarisation by IFNAR-deficient DCs.

Comparison of GMDCs with FLDCs in the *in vitro* CD4^{+} T cell polarisation experiments revealed that GMDCs were much better at inducing IL-4 protein, as well as IL-13 and IL-10 message expression (Fig. 4.12-13 and Fig. 4.19B). These Th2 cytokines were also detectable by ELISA in GMDC cultures (data not shown), as shown previously (Cook *et al.*, 2012), but not in FLDC cultures. One possible explanation for this difference is the fact that FLDCs are more prone to apoptosis than GMDCs (Fig. 4.8 and Fig. 4.18F). Intriguingly, it has also been suggested that IL-2 can inhibit FLDC function, and that FLDCs differentiated in the presence of IL-2 are more susceptible to apoptosis when cultured in the presence of T cells (Lau-Kilby *et al.*, 2011). Thus, it may be the presence of T cells themselves in these cultures that inhibits FLDC function. To address whether this is

the case, we could generate FLDCs using BM from mice that lack subunits of the IL-2 receptor, for example *Il2ra*$^{-/-}$ (CD25-deficient) or *Il2rb*$^{-/-}$ animals, and so cannot respond to IL-2 stimulation (Fontenot *et al.*, 2005; Suzuki *et al.*, 1995; Willerford *et al.*, 1995). It has been shown that GMDCs produce IL-2 at early timepoints after exposure to bacteria, such as *E. coli* (Granucci *et al.*, 2001), and it has been suggested that this is important for T cell activation (Granucci *et al.*, 2001; Zelante *et al.*, 2012). However, whether or not FLDCs produce IL-2 basally or in response to Ag has not yet been addressed. Thus, it would be worthwhile to assess IL-2 production by FLDCs. If FLDCs produce less IL-2 than GMDCs, addition of recombinant IL-2 to *in vitro* T cell co-cultures may enhance T cell survival and polarization, with the caveat that if the findings of Lau-Kilby *et al.* (2011) are correct, then this may affect FLDC survival.

The relatively poor performance of FLDCs in the *in vitro* polarisation assay may also reflect the fact that not all cDCs present in the culture are capable of priming Th2, as FLcDCs include both CD24^{+} and CD11b^{+} cDCs (Naik *et al.*, 2005). There are currently no published studies that address the roles of different cDC subsets in helminth infection. However, our lab is actively investigating this through *S. mansoni* infection of *Batf3*$^{-/-}$ mice, which lack CD8α^{+} cDCs (Alex Phythian-Adams, Angela Marley). A number of studies have been published indicating that CD11b^{+} and monocyte-derived DCs selectively promote Th2 differentiation in mouse models of allergy and airway inflammation (Hammad *et al.*, 2002; Hammad *et al.*, 2010; Plantinga *et al.*, 2013). The importance of mo-DCs in these models is in agreement with our finding that GMDCs are better at polarising Th2 responses than FL-cDCs.

Following the discovery that CD11b^{+} cDCs but not CD8α^{+} cDCs induce Th2 responses in the allergic setting (Plantinga *et al.*, 2013), our working hypothesis is now that this subset may be the FL-cDC population that polarises Th2 responses. We have yet to test whether sorted CD11b^{+} cDCs perform better than the CD8α^{+} subset in the IL-4-dependent *in vitro* polarisation assay. Given that CD11b^{+} cDCs pulsed with SEA express higher levels of co-stimulatory molecules (Fig. 3.A-B) and CCR7 (Fig. 4.2A-C), it is possible that they are the sole DC subset from the FLDC cultures responsible for priming in our adoptive transfer model. This would be difficult to address directly by transferring pure populations of FL-cDC subsets because of the limited cell numbers recovered following FACS sorting.

Despite the issues surrounding FACS-sorting of FLDCs, it is necessary to do these experiments in order to fully address the role of the different DC subsets in SEA-specific Th2 induction. As cell return from flow sorting is so low, this may necessitate several smaller experiments, with fewer recipients per group. This could actually prove to be advantageous, providing a more robust statistical result, if multiple experiments were pooled. Magnetic-bead enrichment (MACS sorting), so far in our hands, has not provided pure enough populations from FLDC cultures, though it may be possible to refine this technique further to enhance purity. MACS sorting would be a preferable method of cell selection because it should be less damaging to cells than FACS. An alternative approach would be to assess cell migration with the dsRed FLDCs and co-staining dLN sections with CD11b or CD24. As described in chapter 3, we have identified clear differences in the responses of $CD8\alpha^+$ FL-cDCs, $CD11b^+$ cDCs and GMDCs to SEA exposure. This raises the possibility that cDC subsets have distinct functions in Th2 responses to schistosomes and other helminth infections.

Since our data clearly shows that *Ifnar1*$^{-/-}$ FL-cDCs do not have any defect in priming T cell responses *in vitro*, this led us to investigate whether their failure to induce *in vivo* Th2 responses is due to their inability to migrate to the dLN. This possibility is supported by the finding that SEA-pulsed *Ifnar1*$^{-/-}$ FL-cDCs, particularly the $CD11b^+$ cDC subset, express much lower levels of CCR7 than WT cells (Fig. 4.14). Some studies have suggested that that IFN-I can influence DC migration, with Mattei *et al* (2009) also identifying reduced expression of Ccr7 mRNA by *Ifnar1*$^{-/-}$ GMDCs, and a resultant impairment in chemotaxis towards CCL21 and CCL19 by these cells in transwell assays. Whilst we were unable to detect any defect in the chemotaxis of *Ifnar1*$^{-/-}$ FL-cDCs in response to CCL21 (Fig. 4.15B), the validity of this experimental model was called into question by the “chemotaxis” of (CCR7-negative) pDCs (Fig. 4.17).

Although chemotaxis assays are often used in studies of cell migration, they are actually one of the more basic measures of *in vitro* cell migration, requiring only that the migrating cell can pass through a 5um pore in a collagen membrane. We based the amount of CCL21 on previous studies (25-250nM; (Wendland *et al.*, 2011)), although much variation was noted in the literature. It may be the case that if we tested a wider range of CCL21 concentrations, we may detect differences in migration, both between the different subsets

of DC, and between WT and *Ifnar1*$^{-/-}$ cells. This assay also requires that cells be maintained in serum-free medium due to the chemotactic potential of FCS, which can negate the effects of exogenous chemokines. Since we found that DCs did not perform well in serum-free RPMI 1640, we instead used X-Vivo^{TM} medium (Lonza) (Fig. 4.15-17). Because of the expense of X-Vivo medium, we initially differentiated DCs in standard RPMI supplemented with 10% FCS, before transfer into X-vivo medium for the chemotaxis assay. This caused a minor increase in cell activation levels, and so the assay may be improved by growing cells in X-vivo from the outset.

Differentiation of human mo-DCs in the presence of IFNα leads to increased CCR7 expression, and this enhances their adhesion to a lymphatic endothelial monolayer, resulting in increased chemotaxis (Rouzaut *et al.*, 2010). This study also found enhanced migration of murine GMDCs differentiated in the presence of IFNα following their injection into the footpad, which was abolished by blockade of the adhesion molecule, LFA-1 (Rouzaut *et al.*, 2010). This suggests that, as well as CCR7 and other chemokine receptors, we should also investigate the expression of adhesion molecules on *Ifnar1*$^{-/-}$ FL-cDCs. Although immobilised CCL21 is required for DCs to adhere to the lymphatic endothelium (Tal *et al.*, 2011), and soluble chemokine gradients direct their movement (Schumann *et al.*, 2010), adhesion molecules facilitate all of these processes. Furthermore, if adhesion molecule expression is greatly reduced on the surface of *Ifnar1*$^{-/-}$ FL-cDCs, we would not be able to detect this defect in our simplistic transwell assay, which does not require adhesion. Adhesion molecules are not only essential for entry of DCs into the dLN, but are also required for strong interactions between DCs and T cells at the immunological synapse (Lim *et al.*, 2012).

Maintenance of leukocytes in the LN is controlled by the signalling sphingolipid S1P and its receptor, S1PR1 (Cyster and Schwab, 2012). Downregulation of S1PR1 on cells prevents egress from the LN, and expression of the receptor is influenced by a number of factors, including the post-translational regulator CD69. Upregulation of CD69 on the cell surface leads to the internalisation and degradation of the S1PR1, inhibiting leukocyte egress (Cyster and Schwab, 2012). CD69 expression is upregulated by IFN-I, and in the absence of IFNAR signalling lymphocyte migration out of the LN is significantly increased (Shiow *et al.*, 2006). Although CD69 upregulation is primarily associated with activated T and B cells,

it has been shown that GMDCs, splenic and Flt3L-generated cDCs and pDCs all upregulate CD69 following exposure to TLR ligands (Alari-Pahissa *et al.*, 2012). It is likely in the absence of IFNAR signalling that CD69 expression would be reduced on FLDCs, which may alter their ability to downregulate the S1PR1, reducing the ability of IFNAR-deficient cells to remain in the dLN. This may have a significant impact on the ability of transferred cells to interact with T cells effectively and to prime Ag-specific responses following transfer. Thus, analysis of adhesion molecules, CD69 and S1PR1 expression by *Ifnar1*$^{-/-}$ FL-cDCs is essential to our understanding of the role of IFN-I for FL-cDC function.

An *in vivo* migration assay would clearly provide the most relevant result for our adoptive transfer model. We do not currently have an *Ifnar1*$^{-/-}$ x dsRed transgenic mouse that would allow us to replicate our experiments assessing WT FLDC migration (Fig. 4.2), although we are in the process of generating this cross. We found that CD45.2 DC transfer into CD45.1 recipients was unreliable (Fig. 4.18), primarily because we could not verify our FACS results with confocal microscopy. The major disadvantage of all adoptive transfer experiments is that a large number of cells must be injected, as very few reach the dLN (around 0.5-5%) (De Vries *et al.*, 2003; Tal *et al.*, 2011; Xu *et al.*, 2007). Although exactly why this is the case is not known, it is likely that, of the cells that are successfully injected, very few make it into the afferent lymphatics (Tal *et al.*, 2011). There is variability inherent in these experiments, as evidenced by our CD45.1/2 transfers (Fig. 4.18). Although we detected clear dsRed^{+} populations following adoptive transfer of FLDCs (Fig. 4.2B), to obtain enough events for FACS analysis it was necessary to pool the dLNs of up to 5 mice in these experiments. As such, it is difficult to draw conclusions about differences in migration kinetics between groups. In order to compare WT and *Ifnar*$^{-/-}$ FL-DC migration using dsRed cells, we will need to use individual mice to give statistical power, which should be possible as these cells are readily detectable by microscopy.

It is possible that our tracking experiments using *Ifnar1*$^{-/-}$ x dsRed FLDCs will demonstrate no migration defect of IFNAR-deficient cells. A possible explanation for the inability of *Ifnar1*$^{-/-}$ FLDCs to induce a Th2 response is that IFNAR signalling in FLDCs is required for sustained Th2 polarisation but not for priming per se. This would explain why we see no defect in an *in vitro* polarisation assay, where Th2 induction is only measured at early timepoints (d3-4). On d7 following *Ifnar1*$^{-/-}$ FLDC transfer we are unable to detect any SEA-

specific Th2 induction. In order to assess whether IFNAR is required for the maintenance of Th2 polarisation rather than priming, we need to assess whether the Th2 response is impaired at earlier timepoints after transfer, around d4-5 following DC injection. Similar published experiments have demonstrated that OX40L is required to maintain SEA-specific Th2 induction but not for the initial priming of the Th2 response (Jenkins *et al.*, 2007). Such experiments would indicate whether IFN-I is required for FLDC survival *in vivo*, or to maintain contact with T cells, as suggested above.

Together, our studies have demonstrated for the first time that cDCs differentiated with Flt3-L can generate SEA-specific Th2 responses following adoptive transfer *in vivo*, and that this Ag-specific polarisation is dependent on the expression of IFNAR (Fig. 4.6). However, GMDCs do not display the same dependence on IFN-I responsiveness for their function (Fig. 4.19A). This difference is likely due to the fact that a much smaller proportion of GMDCs express IFNAR1 on their surface, and at much lower levels (Fig. 4.20). This provides a clear explanation of why this receptor is so important to FLDC function, but not for GMDCs. This then raises the question of why GMDCs express much lower levels of this receptor. Perhaps GM-CSF provides myeloid cells with a survival/activation factor, overriding any requirement for additional activation via IFN-I. This hypothesis is supported by studies demonstrating that stimulation of the GM-CSF receptor itself activates signalling pathways that enhance DC survival and function (van de Laar *et al.*, 2012), as detailed in section 3.3.1. By contrast Flt3 does not display the same capacity to activate these pathways (van de Laar *et al.*, 2012), meaning that FLDCs, and perhaps Flt3-L dependent DC subsets in lymphoid organs, must depend on other signals (including IFN-I) to maintain or amplify their functionality. This may explain why splenic DCs survive less well in culture in the absence of IFNα/β (Mattei *et al.*, 2009). Indeed, it has been postulated that IFN-I is an essential homeostatic factor for controlling signalling pathways and maintaining immune cell function (Gough *et al.*, 2012).

This dichotomy between IFN-I requirements for GMDCs vs. FLDCs raises the interesting question of whether IFN-I has a role in maintaining DC Th2 function *in vivo*, particularly during helminth infection. This may well depend on which DC populations and – perhaps more importantly – which tissues, are studied, at which timepoint in the development of the Th2 response (acute vs. chronic). With this in mind, the next chapter will investigate the

relevance of our *in vitro* findings to the *in vivo* immune response to parasites and parasite antigens.

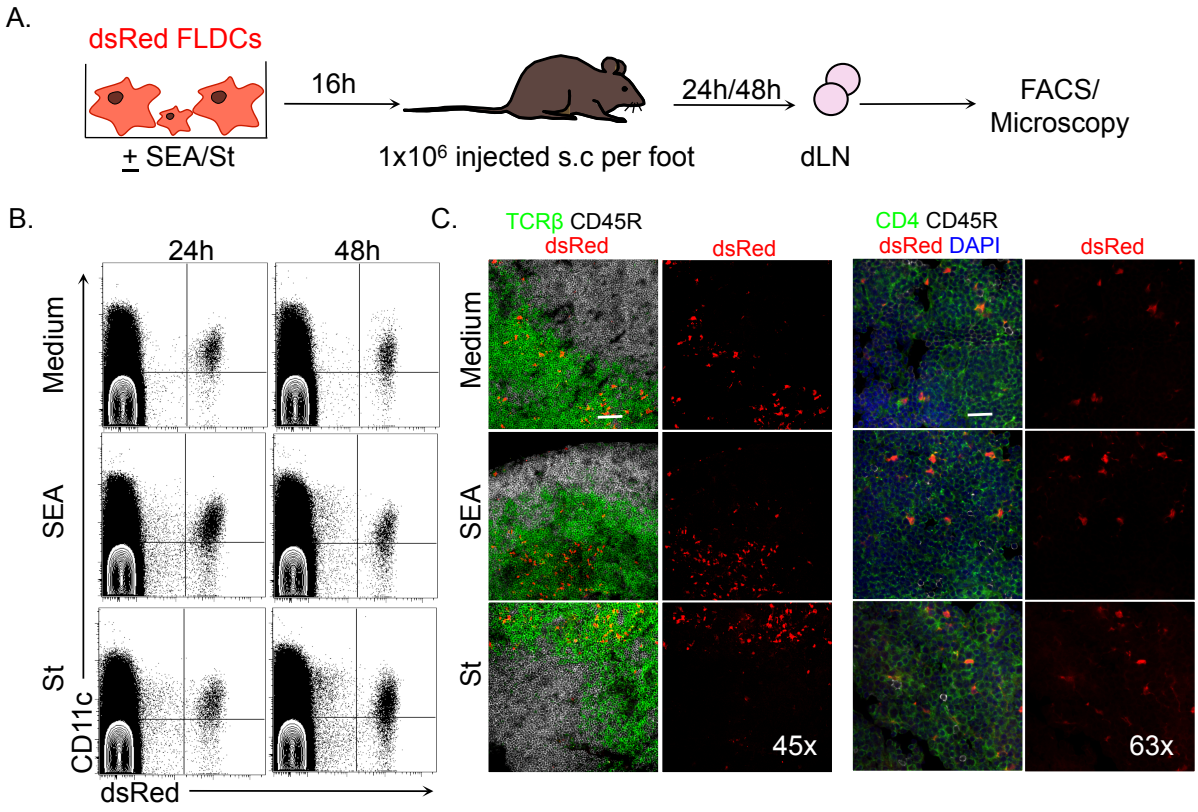


Figure 4.1 FL-cDCs effectively traffic to the draining LN following transfer into naïve recipients.
DsRed FLDCs were stimulated overnight with soluble egg Ags from *S. mansoni* (SEA) or *S. typhimurium* (St), or cultured in medium alone (M), and injected subcutaneously into the feet of naïve recipients on d9. Draining popliteal LNs were harvested 24h or 48h later, stained and analysed for the presence of dsRed$^+$ CD11c$^+$ transferred cells by flow cytometry (B) or by confocal microscopy (C). Left panels in C. depicts 45x magnification of 15μm z-stacks, right panels show 63x magnification. Data representative of 2 independent experiments, plots in B represent pLN cells from 5 mice pooled. Images in C are representative of 3-4 mice per group.

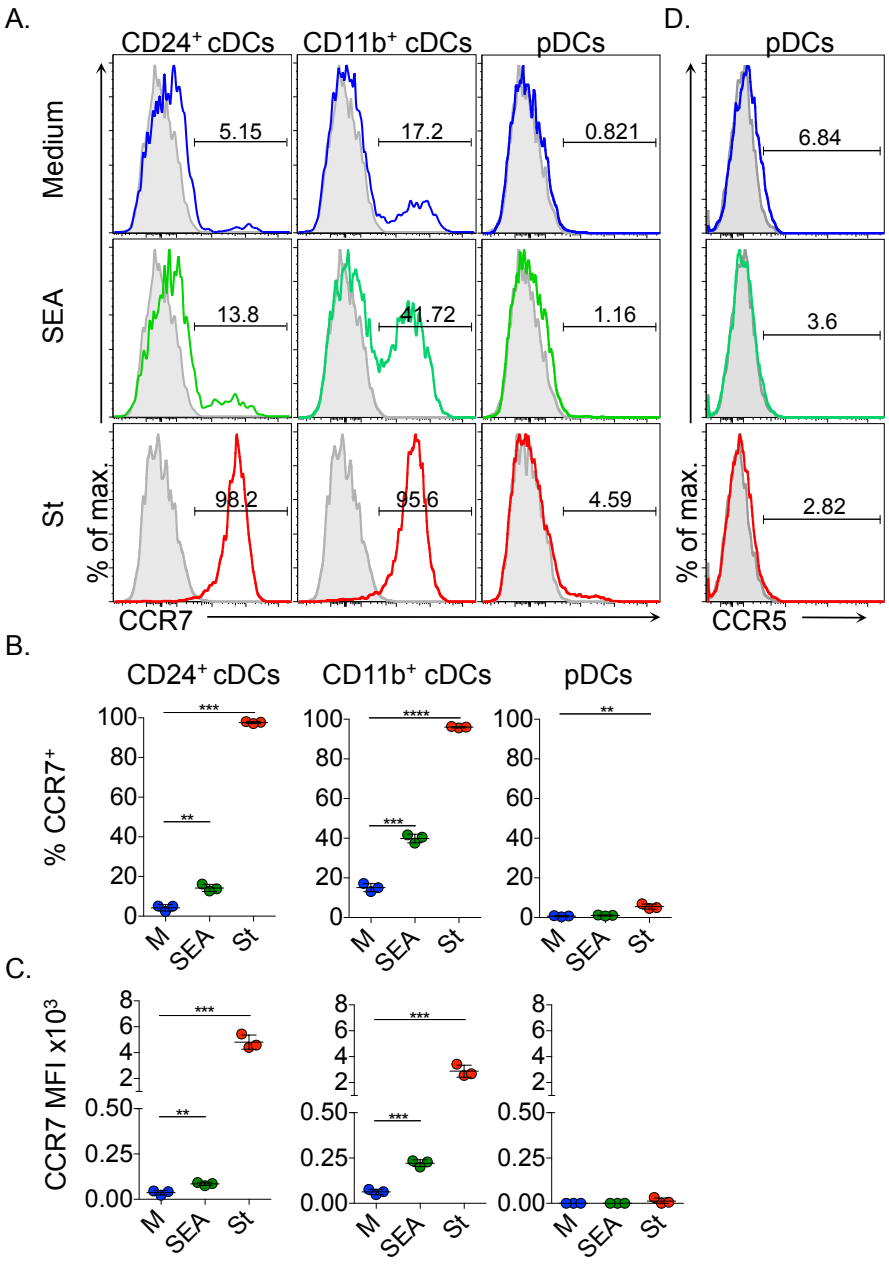


Figure 4.2 FL-cDCs upregulate CCR7 expression on their surface following Ag stimulation.
Cells were cultured overnight with *S. mansoni* soluble egg Ags (SEA), *S. typhimurium* (St) or in medium alone (M), and their expression of chemokine receptors analysed by flow cytometry, histograms of CCR7 (A) and CCR5 (D, pDCs only) expression, gating indicates percentage positive. Percentage of CD24⁺ and CD11b⁺ cDCs, and pDCs that stained CCR7⁺ (B) and gMFI of CCR7 expression on DC subsets (C). Data representative of 4 independent experiments, 3 wells per condition. **P<0.01, ***P<0.001, ****P<0.0001.

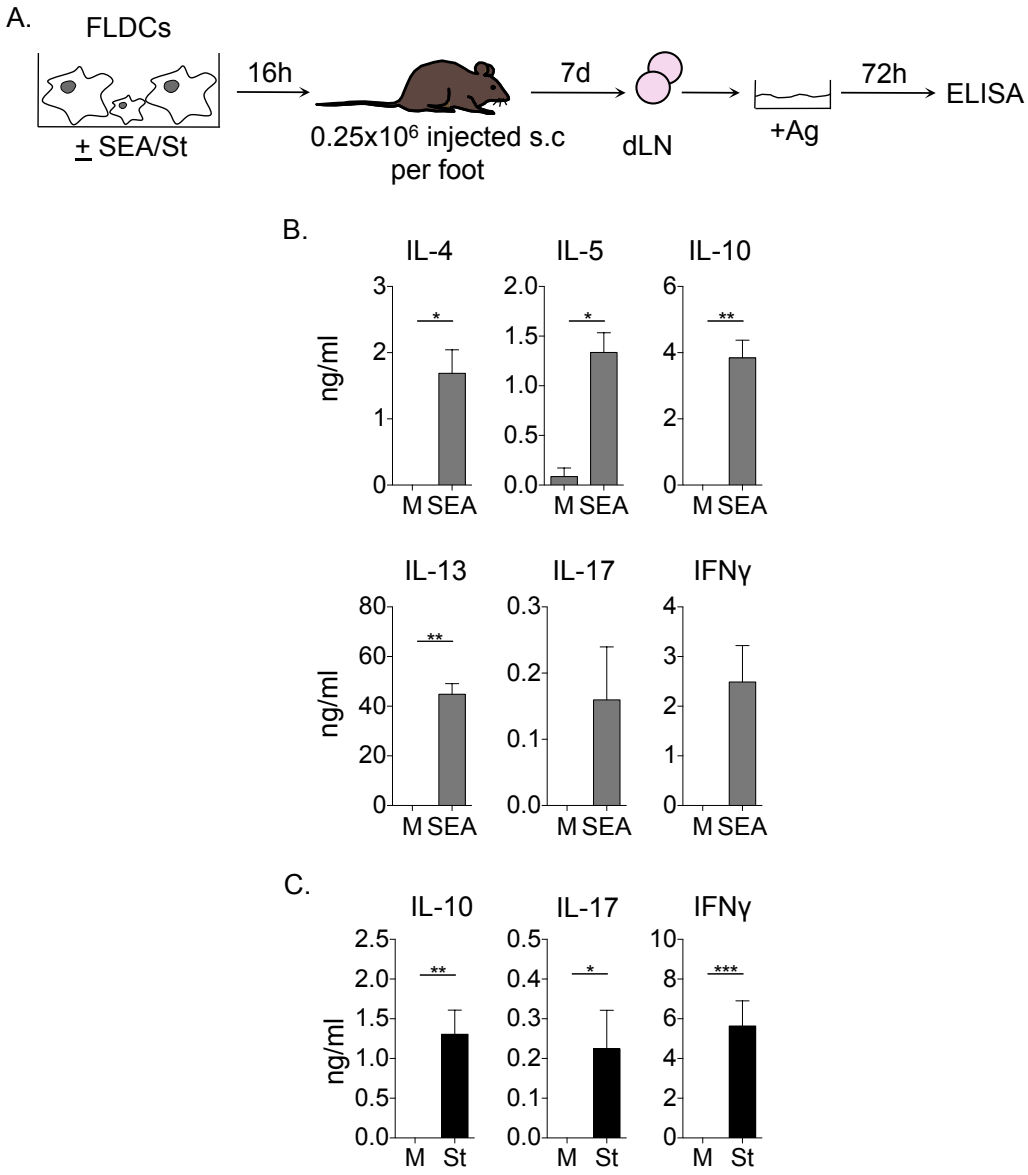


Figure 4.3 FLDCs pulsed with Ag can effectively stimulate Ag-specific T cell responses *in vivo* following transfer to a naïve recipient.
Cells were cultured overnight in medium alone (M), or with SEA (B) or St (C), before being transferred into naïve recipients via subcutaneous injection. On d7 after transfer, the draining popliteal LNs were harvested, dLN cells were plated with/without Ag for 72h. To assess the level of Ag recall response, ELISAs were performed on cell supernatants. Media background subtracted. Data representative of >5 experiments, 5 mice per group. *P<0.05, **P<0.01, ***P<0.001.

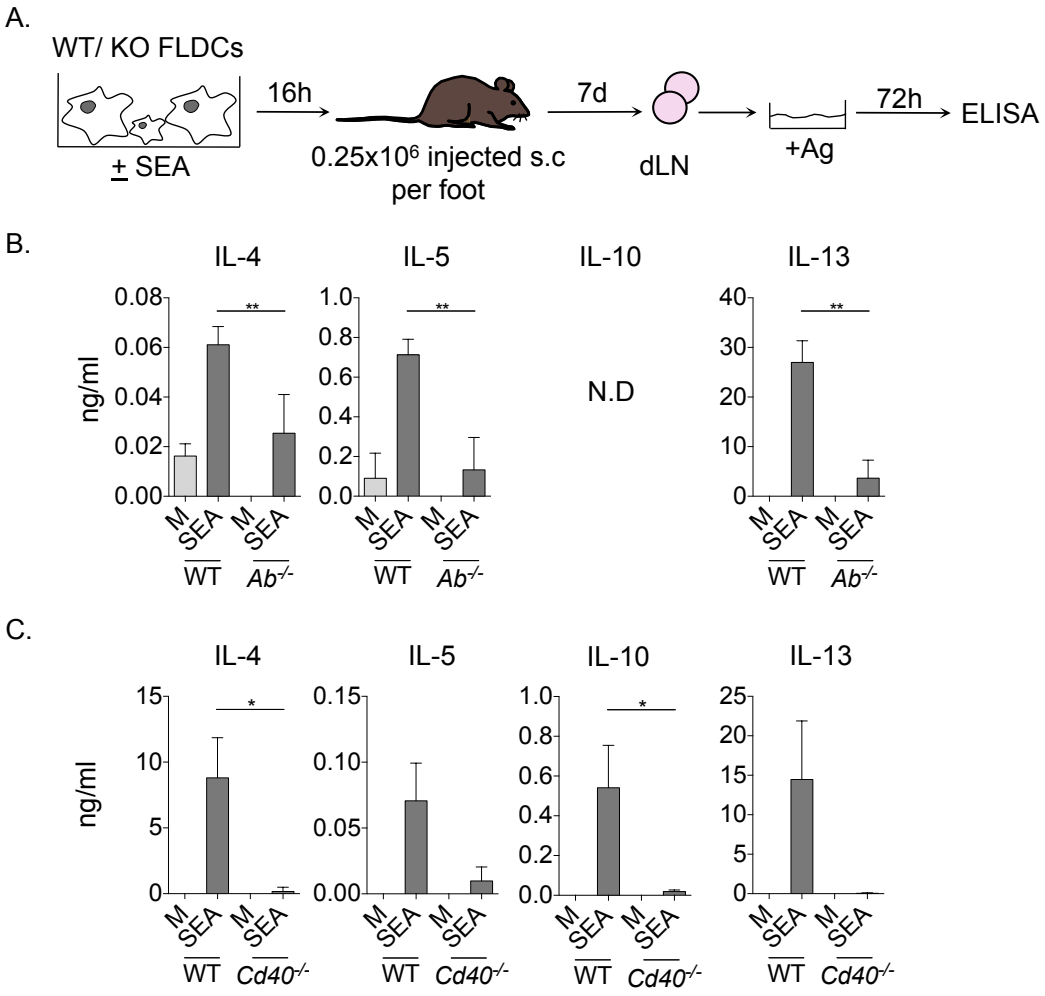


Figure 4.4 Optimal SEA-specific Th2 induction by FLDCs is dependent on their expression of MHC II and CD40.
WT, MHC II-deficient (*Ab*$^{-/-}$ = *H2-Ab*$^{-/-}$, B), or *Cd40*$^{-/-}$ (C) FLDCs were cultured overnight in the presence of SEA, or in medium alone (M), before being adoptively transferred into naïve hosts. dLN were harvested 7d later and restimulated with Ag, cell supernatants were then collected for analysis of T cell cytokine production by ELISA. Medium background subtracted. N.D = None detected. Data representative of 1 (B) and 2 (C) experiments, 5 mice per group. *P<0.05, **P<0.01.

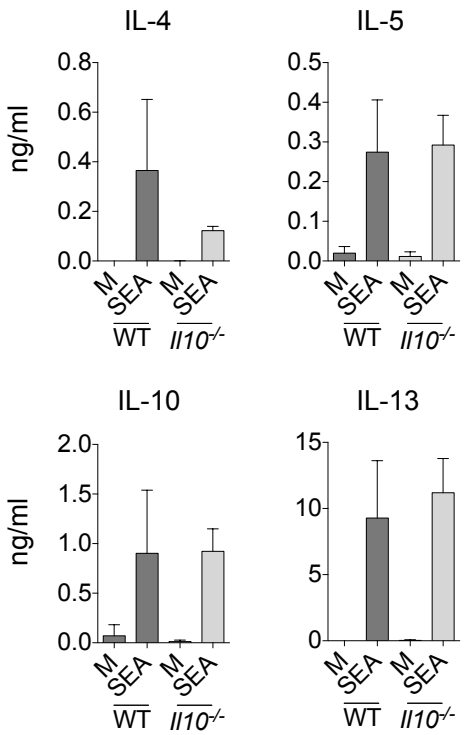


Figure 4.5 SEA-specific Th2 induction by FLDCs does not require DC production of IL-10.
WT or *Il10*$^{-/-}$ FLDCs were cultured overnight in the presence of SEA, or in medium alone (M), before being adoptively transferred into naïve hosts. dLN were harvested 7d later and restimulated with Ag, cell supernatants were then collected for analysis of T cell cytokine production by ELISA. Medium background subtracted. Data from 1 experiment, 5 mice per group.

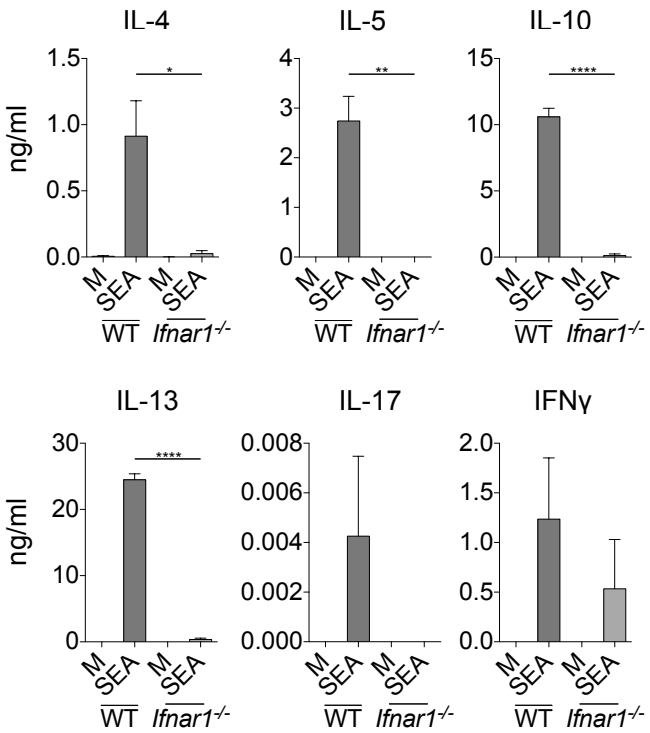


Figure 4.6 FLDCs depend on IFN-I responsiveness for effective SEA-specific Th2 induction.
WT or *Ifnar1*$^{-/-}$ FLDCs were cultured overnight in the presence of SEA, or in medium alone (M), before being adoptively transferred into naïve hosts. dLN were harvested 7d later and restimulated with Ag, cell supernatants were then collected for analysis of T cell cytokine production by ELISA. Medium background subtracted. Data representative of 3 experiments. *P<0.05, **P<0.01, ****P<0.0001.

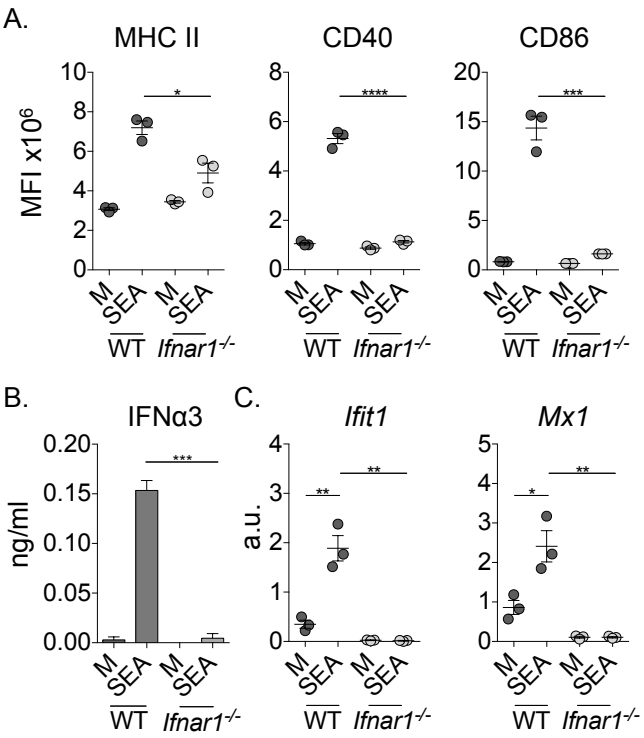


Figure 4.7 FLDC responses to SEA are curtailed in the absence of IRNAR1.
WT or *Ifnar1*$^{-/-}$ FLDCs were cultured for 6h (C) or overnight (A-B) with SEA, or in medium alone (M). Cells were stained and the gMFI of surface marker expression on FL-cDCs analysed by flow cytometry (A). IFNα secretion in cell supernatants was quantified by ELISA (B). RT-PCR was performed on purified mRNA from FLDCs stimulated with Ag for 6h (C). Data representative of 3 experiments, 3 wells per condition. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

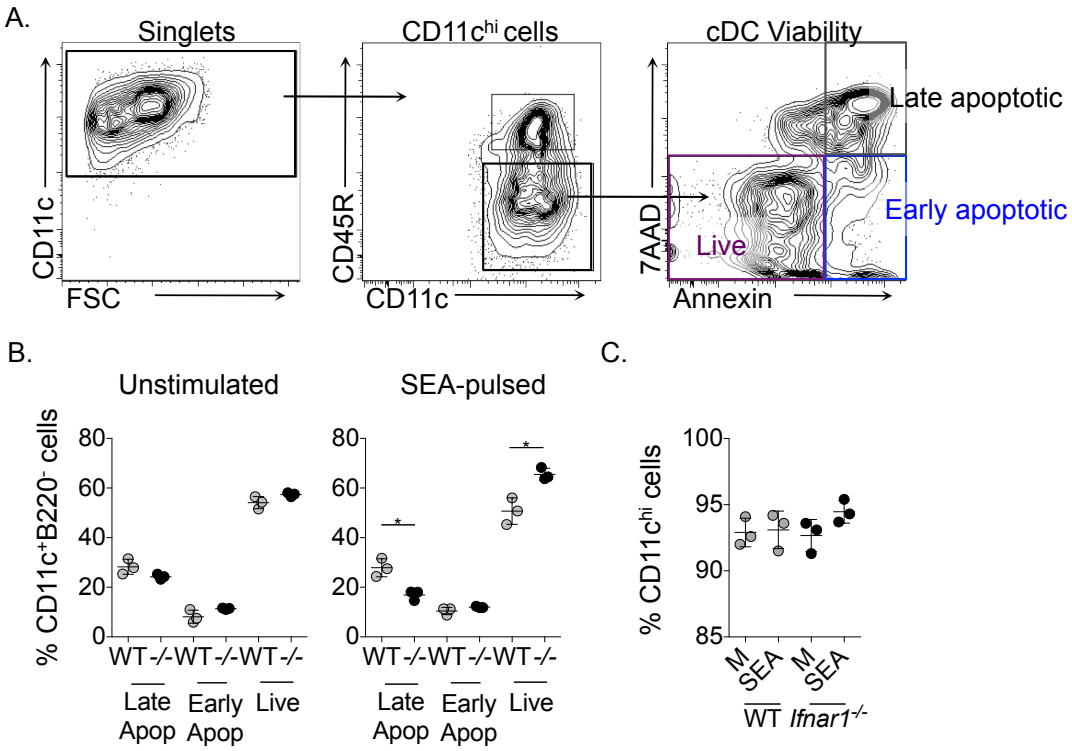


Figure 4.8 *Ifnar1*$^{-/-}$ FL-cDCs display comparable viability and CD11c expression levels to WT cells.
Cells were cultured overnight in the presence or absence of SEA and their viability (A-B) and surface phenotype (C) analysed by flow cytometry. Cells gated on live singlets in C. Data representative of 3 (B) and >5 (C) experiments, 3 wells per condition. *P<0.05.

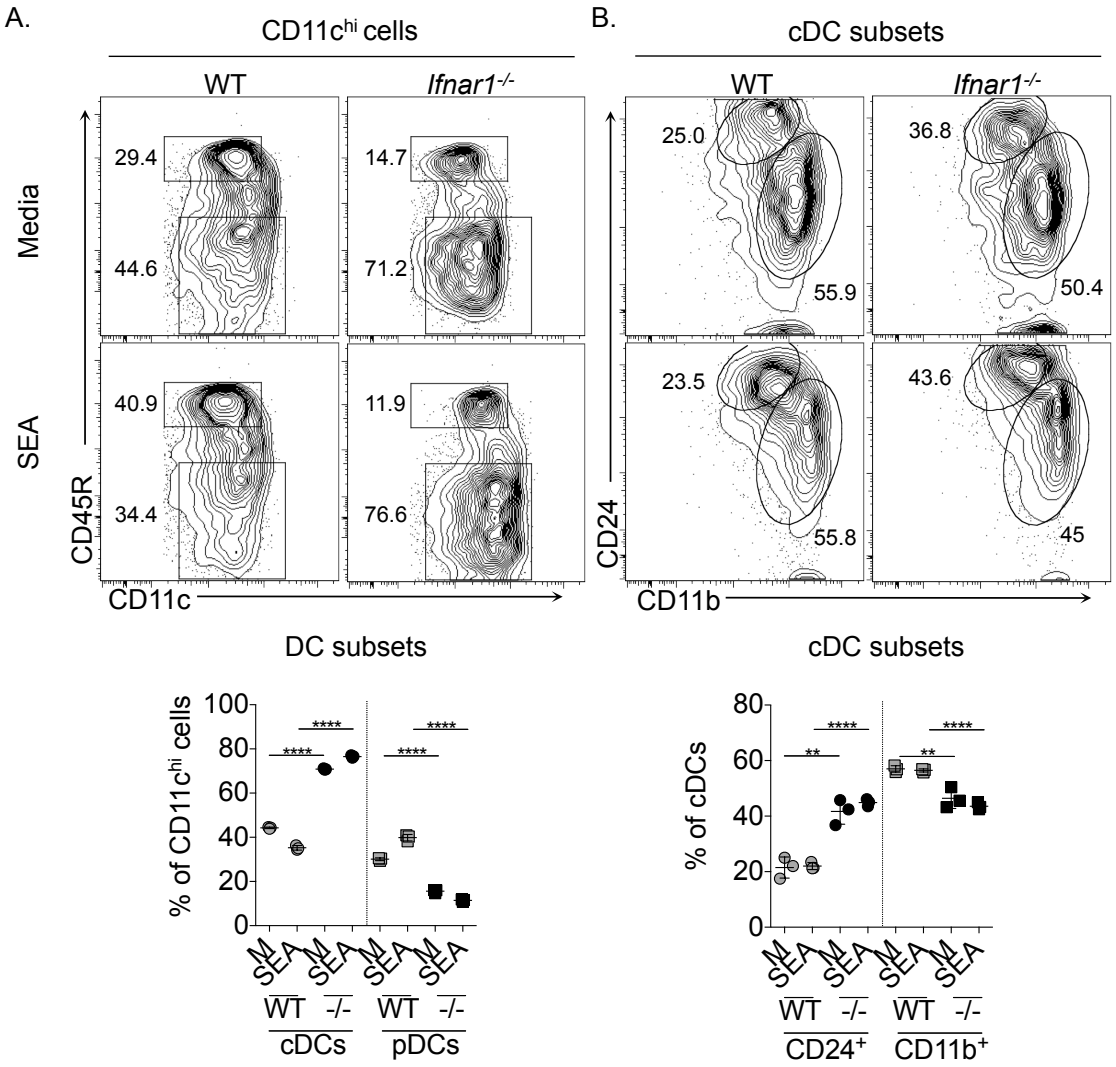


Figure 4.9 *Ifnar1*$^{-/-}$ FLDC mixed cultures consistently contain fewer pDCs and a greater proportion of CD24^{+} cDCs than WT cultures.
WT or IFNAR-deficient FLDCs were cultured overnight in the presence or absence of SEA and the proportions of cell subsets analysed by flow cytometry. Proportions of cDCs (CD11c^{+} CD45R^{-}) and pDCs (CD11c^{+} CD45R^{+}) (A). Proportions of cDC subsets (B): CD24^{+} cDCs (CD11c^{+} CD45R^{-} CD24^{hi} CD11b^{lo}) and CD11b^{+} cDCs (CD11c^{+} CD45R^{-} CD24^{lo} CD11b^{hi}). Gated on live-singlet CD11c^{+} cells. Data representative of 3 experiments, 3 wells per condition. **P<0.01, ****P<0.0001

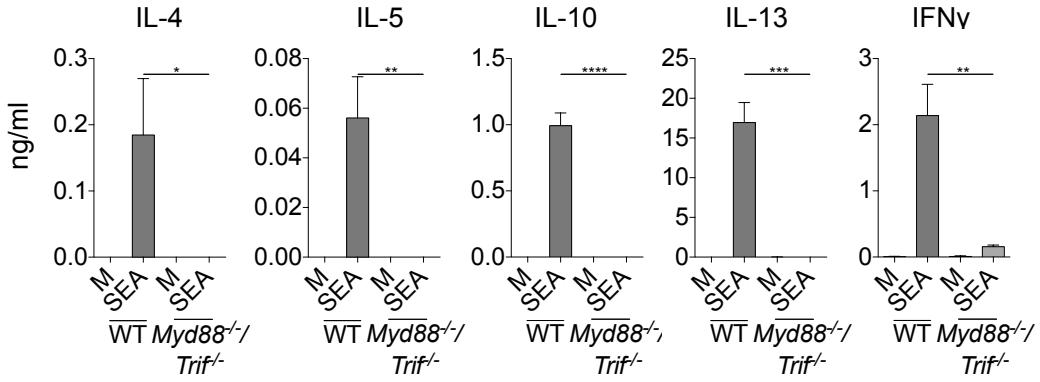


Figure 4.10 Th2 induction by FLDCs is abrogated in the absence of TLR signalling.

WT or *Myd88*$^{-/-}$*/Trif*$^{-/-}$ FL-DCs were cultured overnight in the presence or absence of SEA, before being adoptively transferred into naïve hosts. dLN were harvested 7d later, cells were prepped and restimulated with Ag, cell supernatants were then collected for analysis of T cell cytokine production by ELISA. Medium background subtracted. No IL-17 detected. Data representative of 2 experiments, 5 mice per group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

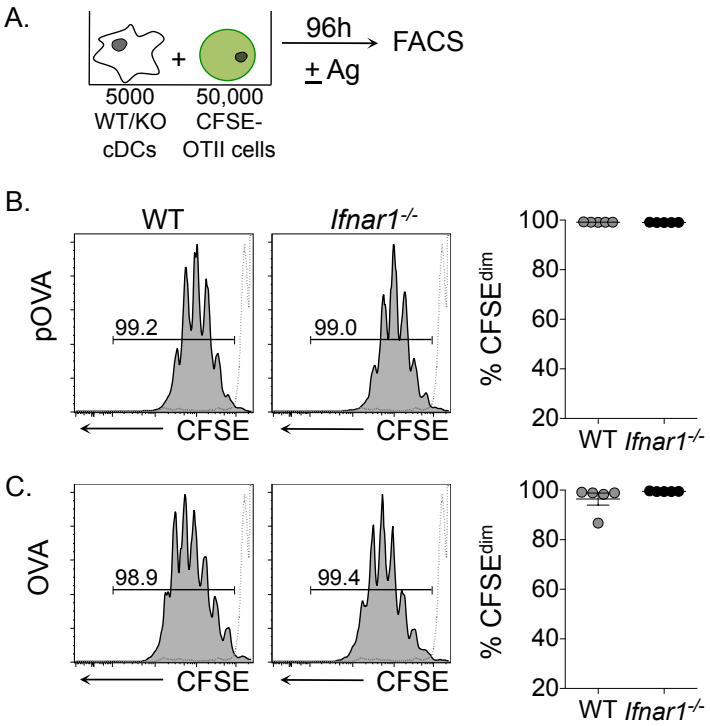


Figure 4.11 *Ifnar1*$^{-/-}$ FL-cDCs can capably present Ag and induce Ag-specific T cell proliferation *in vitro*.
FL-cDCs were sorted on d8 of culture and cultured with CFSE-labelled OTII TCR Tg T cells with either OVA peptide (pOVA, B) or whole OVA protein (OVA, C). On d4 of culture, T cell division was assessed by flow cytometry. Gray dashed line represents division of T cells in the absence of Ag. Data representative of 3 experiments, 5 wells per condition.

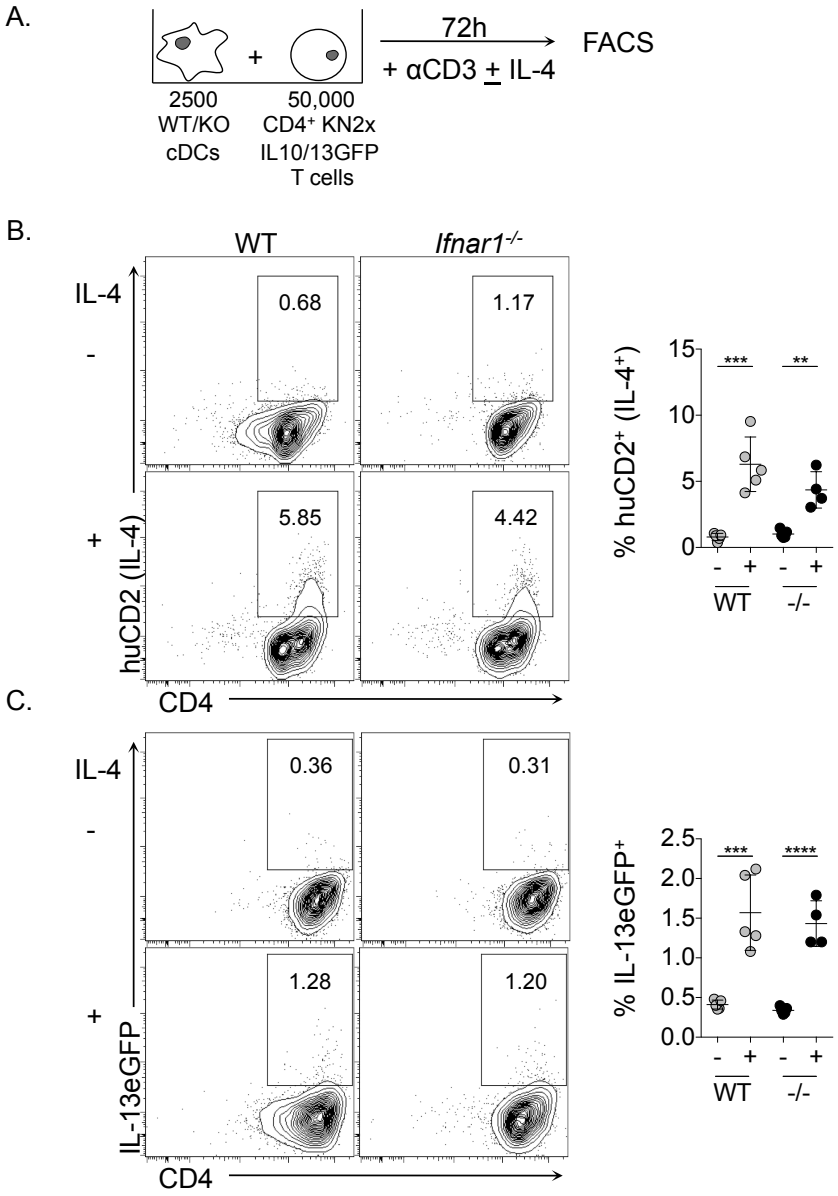


Figure 4.12 *Ifnar1*$^{-/-}$ FL-cDCs can support Th2 polarisation of T cells *in vitro.*

FACS sorted FL-cDCs were cultured with FACS sorted IL-13eGFP^{-}CD4^{+} T cells, in the presence or absence of anti-CD3 and IL-4. HuCD2 (B) and IL-13eGFP (C) expression on CD4^{+} T cells was assessed by flow cytometry after 72h of culture. Data representative of 3 experiments, 4-5 wells per condition. **P<0.01, ***P<0.001, ****P<0.0001.

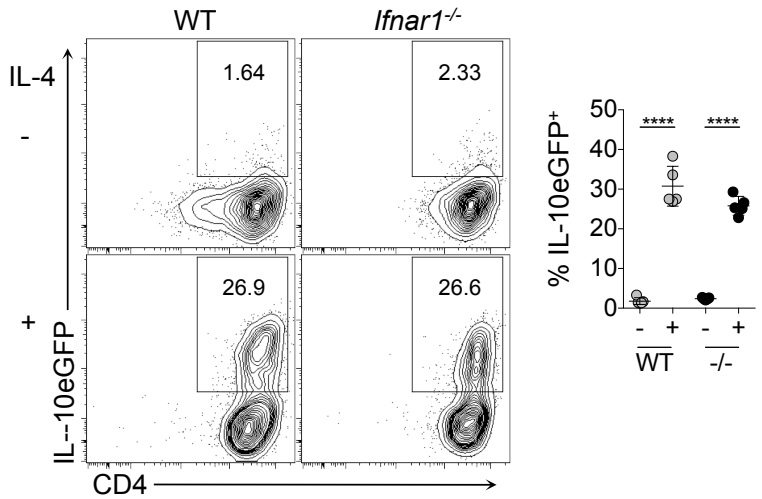


Figure 4.13 *Ifnar1*$^{-/-}$ FL-cDCs can effectively induce IL-10 production in response to IL-4 stimulation *in vitro*. FACS sorted FL-cDCs were cultured with FACS sorted IL-13eGFP^{-}CD4^{+} T cells, in the presence or absence of anti-CD3 and IL-4. IL-10eGFP expression on CD4^{+} T cells was assessed by flow cytometry after 72h of culture. Data representative of 3 experiments, 5 wells per condition. ****P<0.0001.

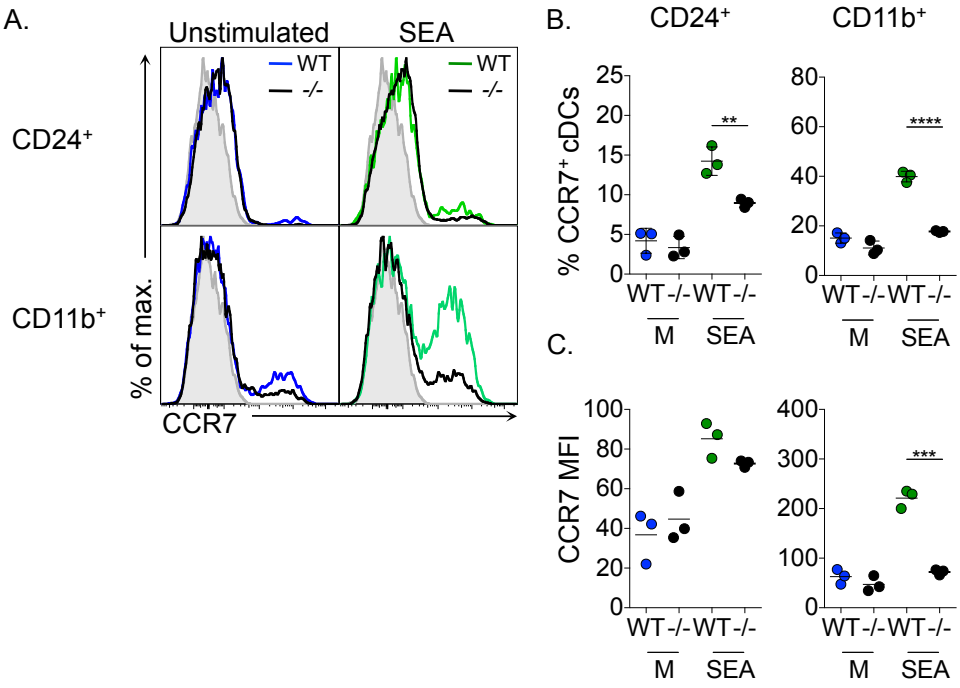


Figure 4.14 In the absence of functional IFNAR, CCR7 upregulation in response to SEA is reduced on FL-cDCs.
WT or *Ifnar1*$^{-/-}$ FLDCs were cultured overnight with SEA, or in medium alone (M), CCR7 expression on CD24^{+} and CD11b^{+} cDCs was analysed by flow cytometry (A). The percentage of CCR7^{+} cells were calculated (B, positive gating as in Fig4.2) and the gMFI for CCR7 recorded (C). Data representative of 3 experiments. *P<0.05, **P<0.01, ****P<0.0001.

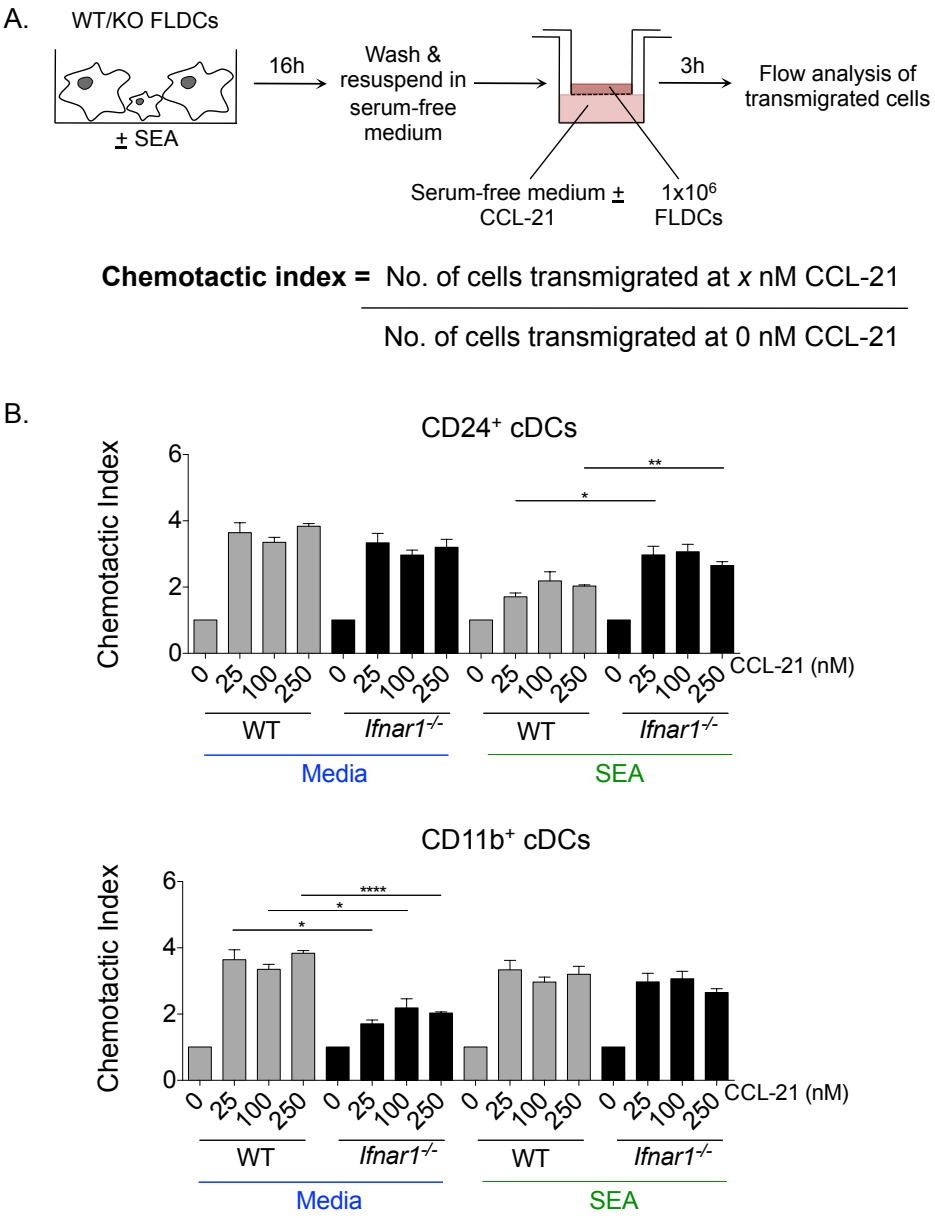


Figure 4.15 Only unstimulated *Ifnar1*$^{-/-}$ CD11b^{+} FL-cDCs show an impairment in an *in vitro* chemotaxis assay.
Following overnight culture in the presence or absence of SEA, FLDCs were washed and resuspended in serum-free medium before being plated in the upper chamber of a transwell. The bottom chamber, separated from the top section by a permeable membrane, contained either 0, 25, 100 or 250nM of CCL-21. After 3h at 37C, any cells in the bottom chamber were collected, stained and counted by flow cytometry. The chemotactic index of the CD8α^{+} and CD11b^{+} cDCs (B) was calculated based on cell counts provided by a MACSQuant. Data from 1 experiment, 3 wells per condition. *P<0.05, **P<0.01, ****P<0.0001.

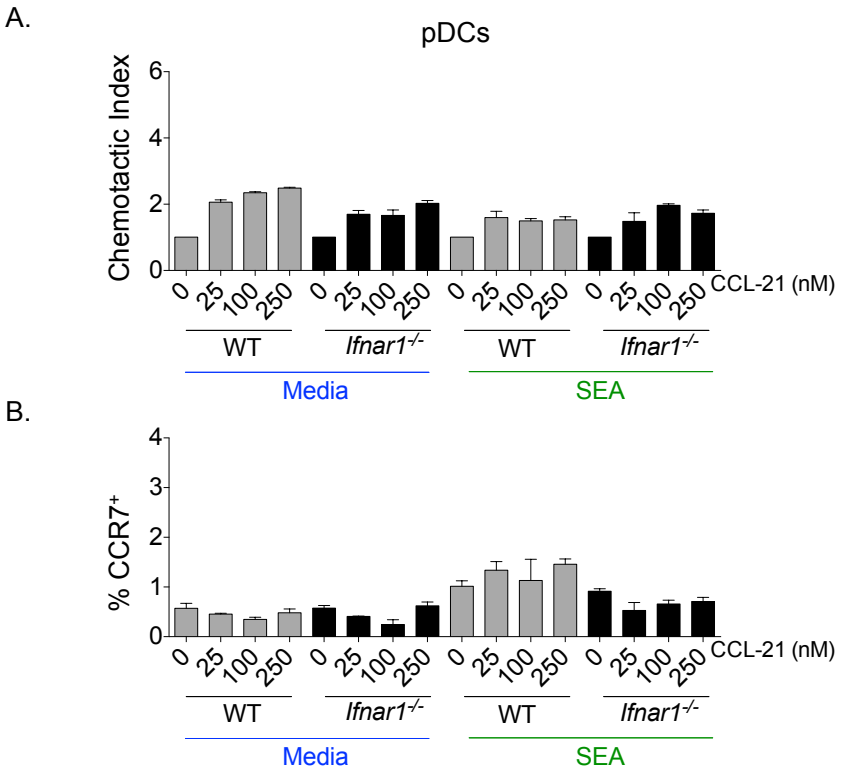


Figure 4.16 FL-pDCs transmigrate in a transwell *in vitro* assay.
FL-DCs were cultured overnight with or without Ag and the transwell assay performed as previously described. Cells in the bottom chamber of the transwell were stained, and cell counts and CCR7 expression analysed by flow cytometry (B). Cell counts were used to calculate chemotactic index (A). Data from 1 experiment, 3 wells per condition.

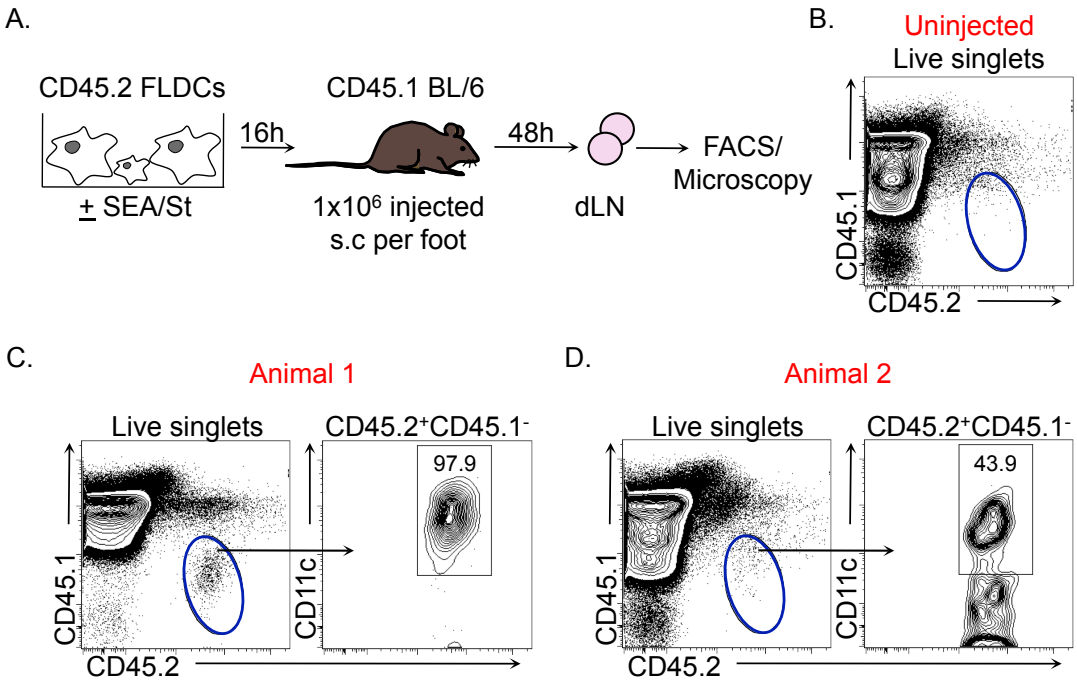


Figure 4.17 Adoptive transfer into CD45.1 recipients is not a sensitive enough method to measure CD45.2 FLDC trafficking to the dLN.
Following overnight Ag stimulation, CD45.2 FLDCs were transferred into naïve CD45.1 recipients. 48h following injection, dLN were harvested and analysed by flow cytometry and microscopy for the presence of $CD45.1^{-}$ $CD45.2^{+}$ $CD11c^{+}$ cells (B-D). Animal 1 (C) and 2 (D) both received transferred cells. Data representative of 3 experiments, 3-4 animals per group.

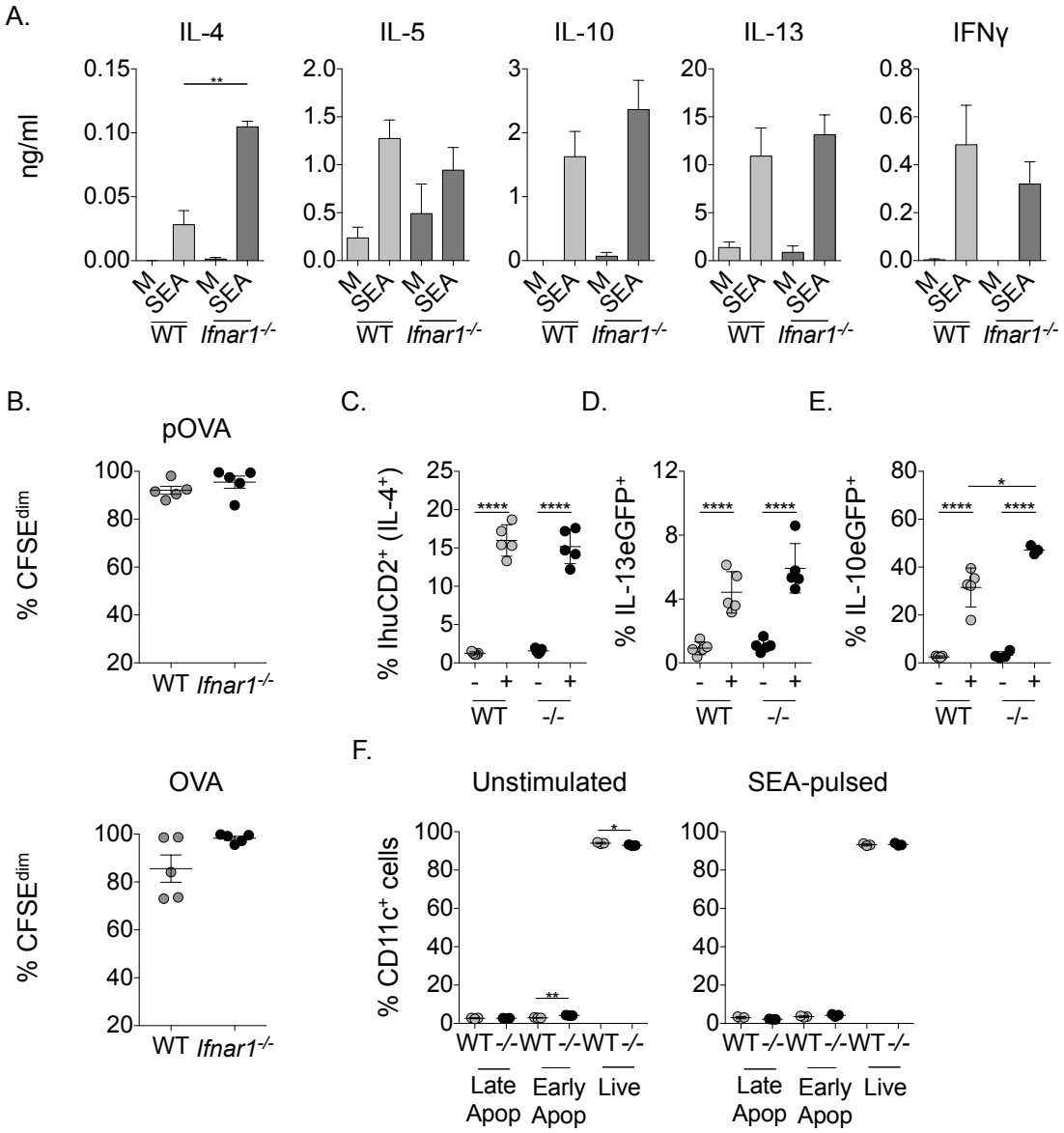


Figure 4.18 GMDCs do not depend on IFNAR expression for their ability to polarise Th2 responses.

GMDCs were cultured overnight with SEA, or in medium alone (M), before being adoptively transferred into naïve recipients. 7d after injection dLNs were harvested and cells were restimulated with Ag. Cell supernatants were collected for analysis of T cell cytokine production by ELISA (A). GMDCs were cultured with CFSE-labelled OTII TCR Tg T cells with either OVA peptide (pOVA) or whole OVA protein (OVA). On d4 of culture, T cell division was assessed by flow cytometry (B). GMDCs were cultured with FACS sorted IL-13eGFP$^-$CD4$^+$ or IL-10eGFP$^-$CD4$^+$ T cells, in the presence or absence of anti-CD3 and IL-4. HuCD2 (C), IL-13eGFP (D) and IL-10eGFP (E) expression on CD4$^+$ T cells was assessed by flow cytometry after 72h of culture. Cells were cultured overnight in the presence or absence of SEA and their viability analysed by staining with Annexin-V and 7-AAD (F). Data representative of 2 (F) or 3 experiments (A-E), 5 animals per group (A) and 3-5 wells per group (B-F). *P<0.05, **P<0.01, ****P<0.0001.

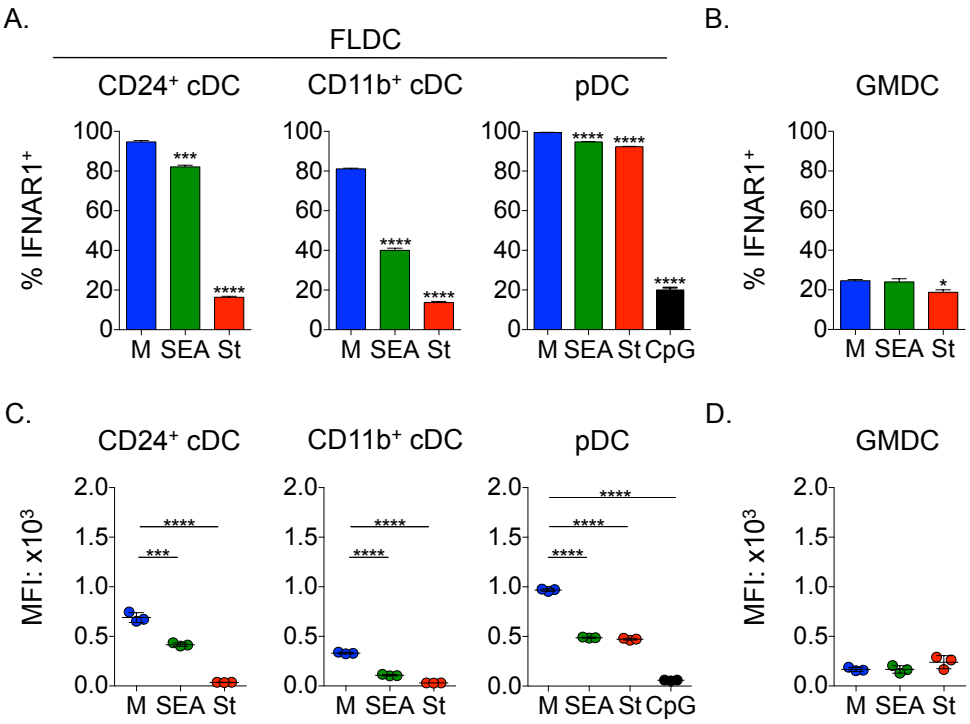


Figure 4.19 Surface expression of IFNAR1 by BMDCs.
BMDCs were cultured overnight in medium (M), with or without SEA or St, cells were then stained and their surface expression of IFNAR1 assessed by flow cytometry. The percentage of cells that were IFNAR1$^+$ (A-B) and IFNAR1 gMFI were calculated (C-D). Data representative of 3 experiments *P<0.05, ***P<0.001, ****P<0.0001. In A. and B., significance value represents significant difference in proportion of IFNAR1$^+$ cells compared to medium condition.

5.0 THE ROLE OF TYPE I IFN IN TH2 RESPONSES AGAINST HELMINTHS

5.1 Introduction

In the preceding chapters, BMDC studies identified an SEA-specific IFN-I signature, suggesting a role for IFN-I in Th2 settings. In agreement with this, we found that IFNAR-deficient Flt3-L-dependent BMDCs were unable to prime SEA responses following adoptive transfer, however, there was no discernible defect in the function of IFNAR-deficient FLDCs *in vitro*. Further, GM-CSF-dependent BMDCs did not share this requirement for IFNAR signalling for induction of SEA-specific Th2 responses. DC development *in vivo* is dependent on Flt3-L (McKenna *et al.*, 2000), but GM-CSF also plays a role in cDC homeostasis in peripheral tissues (Greter *et al.*, 2012; Kingston *et al.*, 2009). Thus it was difficult to predict whether cDC subsets *in vivo* would also depend on IFN-I for optimal Th2 induction.

Given that IFN-I is primarily associated with the activation of Th1, particularly, anti-viral responses, it is intriguing to find that it may play a role in the induction of Th2 responses. So far, however, we have shown only that IFN-I and functional IFNAR are required for SEA-specific Th2 priming by FLDCs. We wanted to expand on this, by investigating the IFN-I signature of DCs challenged *in vivo* with SEA and the development of egg-specific responses in IFNAR-deficient mice. This will provide insights into the relevance of our findings from previous chapters but does not address whether IFN-I is involved in the development, or regulation, of immune responses during active infection.

It is not yet known whether IFN-I is active during helminth infection, or what influence, if any, IFNAR signalling may have on the development of helminth-induced immune responses. Investigating the role of IFN-I and IFNAR during *S. mansoni* infection has implications for our understanding of the processes involved in Th2 induction in this murine model of helminth infection, which has been integral to our understanding of immune processes activated against such parasites that provoke a strong Th2 response, but also elicit severe immunopathology (MacDonald and Maizels, 2008; Perona-Wright *et al.*, 2006b; Phythian-Adams *et al.*, 2010). Perhaps more importantly, such studies also inform our understanding of immune responses during *S. mansoni* infection in humans, an important cause of severe chronic disease in tropical regions (Pearce and MacDonald,

2002; Wynn *et al.*, 2004). To this end, we have begun to examine the role of IFN-I in human DC responses to SEA, as well as investigating whether IFN-I production is detectable during human disease.

Studies examining the role of IFN-I and IFNAR in immune responses during active infection with a range of mircroorganisms, including viruses, bacteria and protozoan parasites, have demonstrated that this cytokine family can enhance immune activation against pathogen attack, but can also have a negative impact on the outcome of infection. *Ifnar1*$^{-/-}$ mice were first characterised for their inability to control viral infection (Hwang *et al.*, 1995; Muller *et al.*, 1994). Since then, IFNAR has been shown to have many roles in antiviral defence, which go beyond the initial induction of intracellular responses. IFNAR signalling is integral to effective adaptive immune activation during viral infection, as functional $CD8^+$ T cell responses are dependent on IFN-I (Aichele *et al.*, 2006; Thompson *et al.*, 2006; Wang *et al.*, 2012). The requirement for IFN-I for optimal $CD8^+$ T cell responses is not always cell-intrinsic, as cross-presentation and priming by $CD8\alpha^+$ cDCs also relies on DC IFN-I responsiveness (Le Bon *et al.*, 2003; Pinto *et al.*, 2011). However, studies of chronic viral infections in IFNAR-deficient mice have shown that IFN-I can also be detrimental to the host, preventing viral clearance in persistent infection, as a result of chronic IFNAR signalling which can limit $CD4^+$ T cell function and IFNγ production (Teijaro *et al.*, 2013; Wilson *et al.*, 2013).

The influence of IFNAR is not limited to antiviral immunity, however, as this pathway also influences immune responses against bacterial and parasite infection. Studies in IFNAR-deficient mice indicate that IFN-I signalling can enhance host resistance to a range of bacteria, including Streptococci species and *Escherichia coli* (Mancuso *et al.*, 2007). However, IFNAR can also act as a hindrance to effective immune activation against intracellular pathogens, inhibiting infiltration or function of innate effectors in *Salmonella*, *Listeria* and *Trypanosoma* infection (Brzoza-Lewis *et al.*, 2012; Chessler *et al.*, 2011; Robinson *et al.*, 2012), and limiting Th1 responses in *Leishmania* infection (Xin *et al.*, 2010). IFN-I clearly impacts on immune mechanisms against microorganisms. However, the role it plays in helminth infections and other Th2 settings has yet to be investigated either in experimental models or in humans.

Although there is no published data on an IFN-I signature from human DCs exposed to helminth Ags, a number of publications have characterised the phenotype of blood DCs from schistosome-infected individuals. Myeloid DC and pDC numbers are reduced in the peripheral blood of *S. haematobium*-infected individuals in endemic areas (Everts *et al.*, 2010a; Nausch *et al.*, 2012). DCs from infected people also express lower levels of HLA-DR (MHC II) on their surface (Everts *et al.*, 2010a), although CD86 and CCR7 expression levels are unchanged compared to cells from control (schistosome egg-negative) individuals (Everts *et al.*, 2010a; Nausch *et al.*, 2012). Myeloid DCs from infected subjects are less responsive to TLR ligands and display poor T cell activating capacity (Everts *et al.*, 2010a), suggesting that DC function is impaired in human schistosomiasis. Like murine BMDCs, human monocyte-derived DCs (moDCs) cultured with SEA produce only very low levels of the inflammatory cytokines IL-6, IL-12p70 and TNFα (de Jong *et al.*, 2002; van Riet *et al.*, 2009). However, SEA-pulsed moDCs upregulate surface expression of OX40L and induce IL-4 production in T cell co-cultures, indicating that similarly to mouse DCs, human cells display only minor phenotypic changes in response to SEA but capably induce Th2 responses (de Jong *et al.*, 2002).

S. mansoni is widely used as an experimental model of human helminth infection for the study of Th2 development and ongoing type 2 inflammation (Allen and Maizels, 2011; Gause *et al.*, 2013; Maizels *et al.*, 2012b), however, we wanted to elucidate whether IFN-I and IFNAR had a similar impact on Th2 responses in other settings. For this reason, we have also examined the immune response of IFNAR-deficient animals during infection with *H. polygyrus*. *H. polygyrus* is an entirely enteric helminth and is used as a model of chronic human hookworm infection (Reynolds *et al.*, 2012). Thus providing an alternative model with which to investigate the role of IFN-I and IFNAR on helminth-elicited immune responses.

5.2 Results

5.2.1 Systemic SEA treatment induces gene expression changes in splenic cDCs

We have found that cDCs generated *in vitro* using Flt3-L have an intermediate level of activation in response to SEA (Section 3.2.2, Fig. 3.2 and Fig. 3.4). Stimulation with SEA also induces an IFN-I signature from FL-cDCs (Section 3.2.3, Fig. 3.3 and Fig. 3.4), characterised by IFNα/β secretion and upregulation of a host of other ISGs (Section 4.2.5, Fig. 4.7). The cDC subsets generated by Flt3-L from murine BM have been shown to share the characteristics of splenic cDCs (Naik *et al.*, 2005). Thus, we hypothesised that splenic cDCs would display a similar phenotype to FL-cDCs following exposure to SEA. To address this, and to examine the relevance of our *in vitro* findings to the *in vivo* setting, we injected mice intravenously with SEA and purified $CD11c^+$ cells from the spleen 12h after treatment (Fig. 5.1A). Splenic cDCs were then FACS-sorted directly into Trizol aliquots, with purity above 95% (Fig. 5.1B-C). We decided to inject mice intravenously as this route of administration delivers Ag systemically, so SEA should reach the spleen relatively quickly. SEA rather than whole *S. mansoni* eggs were used in this model to make it as comparable as possible to the *in vitro* assay. It was difficult to hypothesise at what timepoint after SEA injection we might expect to see a change in surface phenotype or IFN-I production by DCs in the spleen, as we did not know the kinetics of Ag delivery to this site. For this reason we chose to focus on the gene expression profile of splenic cDCs, reasoning that any change in phenotype would be preceded by changes at the mRNA level.

It should be noted that, due to the small numbers of DCs in the spleen and the nature of the purification steps used, spleens from 5 mice were pooled per group. For this reason, we concentrated our analysis on the phenotype of all cDCs, rather than on the individual cDC subsets present in the spleen. In gene expression analysis, each data point represents a replicate from 5-pooled spleens, not individual mice (each replicate being approximately 100,000 cells). Due to the small numbers of cells isolated in this experiment, surface phenotype was not assessed.

We found that SEA treatment induced a significant upregulation of mRNA for CD40 and CD80 (Fig. 5.1D). The transcript for H2-Ab, a subunit of MHC II, was also upregulated 12h after SEA treatment (Fig. 5.1D). No signal for *Ifnα1* or *Ifnβ* was detectable in mRNA from

splenic cDCs of PBS- or SEA-treated mice (not shown). We also did not detect any IFN-I in the serum of mice 12h after SEA injection, or in PBS-treated mice (not shown). However, transcripts for a number of ISGs were upregulated in splenic cDCs at this timepoint, including IFIT1, IFIT3 and MX1 (Fig. 5.1E). The upregulation of these genes suggests that splenic cDCs were activated by systemic SEA treatment and that an IFN-I signature is characteristic of both FL-cDCs and splenic cDCs.

5.2.2 IL-10 and IFNγ production is curtailed in *Ifnar1*$^{-/-}$ mice but the Th2 response is unaffected following subcutaneous injection of *S. mansoni* eggs

Having established that splenic cDCs respond to SEA treatment with a similar phenotype to cDCs generated *in vitro* with Flt3-L, we next wanted to address whether IFNAR is required for the induction of Th2 responses *in vivo*. A model of schistosome-specific Th2 induction is subcutaneous injection of *S. mansoni* eggs into the hind feet of mice (Phythian-Adams *et al.*, 2010). The resulting T cell response is analysed in the draining popliteal LN, and MLNs were taken as a distal LN control. To assess whether IFNAR plays a role in this response, *S. mansoni* eggs were injected into WT C57BL/6 or *Ifnar1*$^{-/-}$ mice and popliteal LNs were harvested 7d after egg injection (Fig. 5.2A). There was no defect in the induction of the Th2 response in *Ifnar1*$^{-/-}$ mice at this timepoint following egg injection as IL-4, IL-5 and IL-13 levels were comparable between WT and IFNAR-deficient animals (Fig. 5.2B). However, there was a significant reduction in IL-10 and IFNγ responses in the dLN of *Ifnar1*$^{-/-}$ mice (Fig. 5.2B).

Whilst the Th2 cytokine response was comparable between groups, there was a significant reduction in cell infiltration into the dLN of *Ifnar1*$^{-/-}$ animals following egg injection (Fig. 5.2C), suggesting a defect in immune activation. Analysis by flow cytometry revealed that the proportion of TCRβ^{+} cells in the dLN of IFNAR-deficient mice was significantly increased compared to WT (Fig. 5.2D); consequently, the percentage of CD4^{+} T cells was also elevated (Fig. 5.2D). However, the proportion of Foxp3^{+} CD25^{+} Tregs in *Ifnar1*$^{-/-}$ dLNs was not significantly altered (Fig. 5.2D). An increased percentage of CD4^{+} T cells in the dLN would mean that a greater number of these cells were present in culture wells during Ag restimulation, which could impact on the cytokine readout. For this reason, cytokine concentrations were adjusted to establish the amount secreted per 100,000 CD4^{+} T cells (Fig. 5.2E), providing a fairer comparison of cytokine production by WT and IFNAR-

deficient mice. This analysis suggested that, as well as IL-10 and IFNγ, IL-13 production was also significantly reduced in *Ifnar1*$^{-/-}$ dLNs (Fig. 5.2E). These findings indicate that while IFNAR is required for optimal T cell responses following egg injection, it is not essential for Th2 induction in this model.

5.2.3 IFN-I is elevated in the serum only at high doses of *S. mansoni* infection

In order to assess whether IFN-I was elevated systemically during infection, we measured cytokine levels in the serum of animals on d56 after infection with different doses of *S. mansoni* cercariae (Fig. 5.3A). No IFNβ was detected in the serum of any mice in this experiment and IFNα3 levels were not significantly increased at any dose of infection (Fig. 5.3B). 2 out of 4 animals infected with 160 cercariae did have elevated systemic levels of IFNα3 compared to naïve mice. However, the other mice in the 160 cercariae group did not share this phenotype, thus there was no statistically significant change in IFNα levels at this dose. The wide spread of the data in the 160 cercariae group demonstrates that more repeats are needed, and a larger sample size would enhance the statistical power of this analysis, this is the case for all cytokines analysed at this dose. Of the Th2-associated cytokines, IL-4 levels were significantly elevated at 40, 80 and 160 cercariae dose (Fig. 5.3B). IL-13 was also above background levels in mice infected with 40 and 80 cercariae, but not at the 160 dose. However, IL-5 and IL-10 levels were not altered compared to naïve controls. The inflammatory cytokines also failed to show a consistent pattern, with no change in systemic IL-6 at any dose of infection, IL-12/23 p40 was elevated only at 80 cercariae dose. IFNγ and IL-17 levels were above naïve at 20 and 80 cercariae dose, however these cytokines were more variable at the other doses of infection and so were not significantly elevated.

These data were suggestive of elevated IFN-I at high doses of *S. mansoni* infection. However, we had not sampled enough animals to assess IFN-I reliably in this experiment, given the clear variability in the serum cytokine data within the 160 cercariae group. For this reason, we analysed the serum cytokine levels of a larger cohort of animals with high intensity infection (180 cercariae – Fig. 5.4A). These mice were killed on d49 because, at higher doses, animals can start to lose weight and may die before reaching d56 of infection. IFNα3 levels were elevated in the serum of 75% of infected mice, and the increase compared to naïve animals was highly significant ($p=0.0001$, Fig. 5.4B). Systemic

levels of IL-5 ($p<0.0001$) and IL-12p40 ($p<0.0001$) were also similarly increased in the serum of infected mice (Fig. 5.4B). IL-10, IL-17 and IFNβ levels were also significantly higher than in naïve serum, although these cytokines were at baseline in the majority of infected animals (Fig. 5.4B). IFNγ, TNFα, IL-4, IL-6 and IL-12p70 were not significantly elevated in the serum of mice on d49 following infection with 180 *S. mansoni* cercariae. Systemic IFNα levels from infected mice were compared with the IL-5 and IL-10 levels in the same samples; however, there was no correlation between an increase/ decrease in Th2 or regulatory cytokines and an increase in systemic IFNα (Fig. 5.4C).

5.2.4 The role of IFNAR in the immune response against helminth infection

We found that systemic IFNα/β levels were only significantly elevated at very high doses of *S. mansoni* infection. However, IFN-I production is often difficult to detect because IFN-Is are often secreted at relatively low-levels, or for a short period of time, and are taken up by a variety of cell types promptly following secretion. Our work with Flt3-L-dependent BMDCs suggests that IFNAR expression may be required for efficient priming of Th2 responses (Chapter 4, Fig. 4.6). In *S. mansoni* infection, a dominant Th2 response is induced in response to egg deposition, which begins at around d28 of infection (Pearce and MacDonald, 2002). The Th2 response is detectable in infected tissues and draining LNs, including the liver and MLNs, from around d42 of infection (Grzych *et al.*, 1991; Pearce *et al.*, 1991). One of the most affected tissues is the liver because the majority of parasite eggs are swept into the hepatic blood system (Wynn *et al.*, 2004). With IFN-I transcripts upregulated in the liver at d42 of infection with 40 cercariae (Fig. 5.5B, PCR performed by Rachel Lundie), this may suggest a role for IFNAR in the priming of T cell responses at this stage of schistosome infection. To address this, WT or *Ifnar1*$^{-/-}$ mice were infected with 40 *S. mansoni* cercariae and sacrificed on d42 of infection. This infection dose was chosen to ensure that this experiment was comparable to the timecourse infection study described above. Mouse weights were recorded from d35 of infection. The weights of both WT and *Ifnar1*$^{-/-}$ animals on d37 were reduced compared to d35 levels, with IFNAR-deficient mice losing slightly more weight. However, by d40 the weight change of all animals was comparable (Fig. 5.6B). Animal weights were assessed only for a short period during infection, thus a more extensive assessment of weight change is required over the course of infection to confirm no cachexia in IFNAR-deficient animals.

Before harvesting tissues, mice were perfused by injecting PBS into the heart, to expunge all blood from the organs. This process also flushes out most of the schistosome worms resident in the blood system. The perfusate was collected from infected animals and the number of adult worms was assessed. *Ifnar1*$^{-/-}$ animals had significantly fewer female worms than WT mice (Fig. 5.6C). However, although 3 of the IFNAR-deficient mice had no female worms in the perfusate (Fig. 5.6C), they were patently infected because schistosome eggs were detectable in all liver samples, and all but one gut sample (Fig. 5.6D). This illustrates that manual perfusion is not successful at recovering all worms. Although there was a slight reduction in liver and gut egg counts in *Ifnar1*$^{-/-}$ mice, this did not reach statistical significance (Fig. 5.6D). This preliminary data will be bolstered by further repeats involving more animals, in order to ascertain if there is any consistent change in worm (and egg burden) or whether this result is merely a reflection of the inaccuracy inherent in the perfusion technique.

Hepatomegaly and splenomegaly provide a general measure of pathology during schistosome infection. Both WT and IFNAR-deficient animals had significant hepatomegaly and splenomegaly, and there was no significant difference between infected groups (Fig. 5.6E). Thus, overall the data suggests no major difference in parasitaemia or pathology in infected IFNAR-deficient animals.

Cells were purified from the liver and MLN of all mice and cultured with SEA in order to assess the recall response to egg Ags. In the liver, only SEA-specific IL-10 was significantly reduced in IFNAR-deficient mice; levels of IL-4, IL-5, IL-13 and IFNγ were equivalent between WT and *Ifnar1*$^{-/-}$ animals (Fig. 5.7B). Both IL-4 and IL-13 levels were significantly reduced in the MLN (Fig. 5.7C), whilst IL-10 and IL-5 were not significantly altered in the MLNs of infected *Ifnar1*$^{-/-}$ mice (Fig. 5.7C). Similarly to the liver, there was no change in IFNγ levels in the MLN of IFNAR-deficient animals (Fig. 5.7C). No IL-17 was detected from liver or MLN cells.

Liver and MLN cell counts were comparable in both infected groups (Fig. 5.8A). However, MLNs were significantly smaller in naïve *Ifnar1*$^{-/-}$ animals (Fig. 5.8A). The percentage of $CD4^+$ T cells present in each sample was calculated as the proportion of all live intact-

singlets that were TCRβ$^+$ CD4$^+$ (using event counts from FACS data). To quantify the total number of CD4$^+$ T cells in the liver or MLNs, the number of viable leukocytes was multiplied by the percentage of T cells as determined by flow cytometry. This analysis demonstrated that there was no significant alteration in the proportion, or actual number, of CD4$^+$ T cells present in the liver or MLN of *S. mansoni* infected IFNAR-deficient animals (Fig. 5.8B-C). Further analysis of cell populations in these tissues indicated no significant differences in the proportions or total numbers of B cells (CD19$^+$ B220$^+$) or Treg (TCRβ$^+$ CD4$^+$ Foxp3$^+$ CD25$^+$) populations between infected groups (Fig. 5.8B-C). There may be a reduction in lymphocyte populations in the livers of naïve IFNAR-deficient mice. However, this needs to be assessed with a larger number of animals to allow for statistical analysis (Fig. 5.8B-C). Together, these data indicate that IFNAR was required for optimal Th2 responses in the MLN on d42 of *S. mansoni* infection. In the liver, only IL-10 induction appeared to require IFNAR.

Up to this point, all of our experiments had focused on the role of IFNAR in the induction of immune responses to schistosomes. To assess whether IFN-I signalling has an important function in other helminth settings, we infected WT or *Ifnar1*$^{-/-}$ mice with the murine gastrointestinal nematode parasite *H. polygyrus*. Infected groups had comparable egg counts on d14 and d21 of infection; however, d28 egg counts were significantly higher in the faeces of IFNAR-deficient mice (Fig. 5.9B). There was no significant difference in worm burden between infected groups on d28 (Fig. 5.9C). An increase in egg burden is a phenotype associated with a deficiency in the Th2 response to *H. polygyrus* (Maizels *et al.*, 2012a). Therefore, it was not unexpected to find that production of Th2 cytokines was reduced in the MLN of *Ifnar1*$^{-/-}$ mice on d7 of infection (Fig. 5.9D). IL-4 and IL-5 were significantly reduced, whereas IL-13 production was unaffected (Fig. 5.9D). Although the Th17 response was comparable between infected groups, Th1 induction was impaired in IFNAR-deficient animals, with a significant reduction in IFNγ levels in this group (Fig. 5.9D). There was no significant difference in IL-10 between infected groups (Fig. 5.9D). In combination with the preliminary data from *S. mansoni* infection, it seems reasonable to conclude that IFNAR signalling is required for optimal induction of adaptive immune responses in helminth infection, particularly in the MLN, with some facets of the Th2 response being more dramatically affected than others in the global absence of IFNAR.

5.2.5 Investigating the role of IFN-I in human responses to *S. mansoni*

Having found that SEA treatment induces an IFN-I signature from murine Flt3-L-dependent cDCs in the spleen (Fig. 5.1E), as well as *in vitro*-generated FL-cDCs (Fig. 3.3B), we wanted to address whether any hallmarks of this response were detectable from human DCs following exposure to SEA. Bart Everts (University of Washington, USA) performed the purification and stimulation of human DCs for us. The DC subsets present in human blood – pDCs, $CD1c^+$ cDCs (comparable to murine $CD11b^+$ cDCs (Robbins *et al.*, 2008)) and $CD141^+$ cDCs (equivalent to $CD24^+$ FLDCs/ splenic $CD8\alpha^+$ cDCs in mice (Jongbloed *et al.*, 2010)), were purified from samples supplied by donors from a non-endemic area (U.S.A) and cultured overnight with SEA or TLR ligands (Fig. 5.10A). pDCs were stimulated with CpG, which is recognised by TLR9, as human pDCs express very high levels of this receptor and produce large quantities of IFN-I in response to exposure to this ligand (Hemont *et al.*, 2013). As a positive control, $CD1c^+$ DCs were stimulated with R848, a synthetic TLR7/8 ligand, having previously been reported to express TLR7/8 and to secrete IFNα following exposure to this Ag (Meixlsperger *et al.*, 2013; Piccioli *et al.*, 2007). $CD141^+$ DCs express high levels of TLR3 and produce IFN-I in response to pI:C, a TLR3 ligand (Jongbloed *et al.*, 2010; Poulin *et al.*, 2010), hence, this Ag was used as a positive control for this subset. Mo-DCs were also generated from monocytes isolated from these samples and stimulated with SEA or CpG. Mo-DCs have previously been shown to express TLR9 and produce IFNα in response to CpG (Hoene *et al.*, 2006).

Supernatants were shipped to us and we assessed the cytokines produced by these cells after overnight culture in the presence or absence of Ag. IFNβ production was not assessed, as the reagents for IFNβ quantification are prohibitively expensive. However, the assay used to measure IFNα detects all 12 human IFNα subtypes. No IFNα was detectable in the supernatants of any of the DC subsets cultured with SEA, either with cells directly purified from the blood, or from human mo-DCs generated from the same donor (Fig. 5.10B). IFNα was detected only in supernatants from pDCs responding to CpG (Fig. 5.10B). Human mo-DCs did not produce detectable levels of any other innate cytokines (TNFα, IL-1β, IL-6, IL-10 and IL-12p70) in response to SEA stimulation (Fig. 5.10B).

We did not detect any IFNα production from $CD141^+$ DCs in response to pI:C, despite published reports of IFN-I production from this subset following pI:C exposure (Hemont *et al.*, 2013; Jongbloed *et al.*, 2010; Meixlsperger *et al.*, 2013). However, it is in line with the findings of Jongbloed *et al*. (2010), who detected IFNβ secretion in response to pI:C, but not IFNα. No R848-induced IFNα was detected from $CD1c^+$ DCs, however, this TLR7/8 ligand stimulated high-level production of TNFα, IL-1β, IL-6, IL-10 and IL-12p70, as previously reported (Hemont *et al.*, 2013) (Fig. 5.10B). IFNα secretion was also not detected from CpG-stimulated mo-DCs despite previous reports of IFN-I production by human mo-DCs in response to this ligand (Hoene *et al.*, 2006) Fig. 5.10B).

To our knowledge, information on the levels of IFN-I in *S. mansoni*-infected individuals has not been published. For this reason we analysed the serum IFNα levels of a sample of people who were part of a cohort from an endemic area in Kenya (samples from David Dunne, University of Cambridge). We had found that in a mouse model of *S. mansoni* infection, serum IFNα was elevated only at high doses of infection (Fig. 5.3). Thus, IFNα levels in the serum of individuals who had medium or high egg burden by faecal counts were compared to the serum levels of egg-negative individuals (Fig. 5.11A). There was no correlation between faecal egg counts and the amount of IFNα detected in the serum (Fig. 5.11A). It is known that resistance to *S. mansoni* infection increases with age (Pinot de Moira *et al.*, 2010) and the median age of egg-negative individuals was higher than those with high faecal egg counts. However, there was no correlation between IFNα levels in the serum and the age of individuals sampled (Fig. 5.11B).

5.2.6 Summary:

- Splenic cDCs upregulated ISGs at the messenger RNA level in response to systemic SEA treatment (Fig. 5.1E)
- Regulatory and Th1 cytokine production was reduced in the dLN of *Ifnar1*$^{-/-}$ mice following schistosome egg injection, whilst facets of the Th2 response were also downregulated (Fig. 5.2B & E).
- In a mouse model of *S. mansoni* infection, IFN-I could be detected in the serum at high levels of infection intensity (Fig. 5.4B)
- IFNAR-deficient mice display reduced Th2 cytokine responses in the MLN during *S. mansoni* (Fig. 5.7B) and *H. polygyrus* (Fig. 5.9D) infection
- IFNα was not detectable in the supernatants of human mo-DCs or purified blood-borne DCs following SEA stimulation (Fig. 5.10B)
- Serum levels of IFNα were not significantly altered in *S. mansoni* infected individuals (Fig. 5.11)

5.3 Discussion

Work presented in chapter 4 illustrates that expression of IFNAR is necessary for SEA-specific Th2 induction by Flt3-L dependent BMDCs but is dispensable for, and may even inhibit, optimal Th2 induction by BMDCs generated using GM-CSF (Fig. 4.8A and Fig. 4.8E). Although Flt3-L is an essential growth factor for DC differentiation *in vivo* (McKenna *et al.*, 2000), and FLDCs represent DC subsets found in lymphoid tissues in the steady state (Naik *et al.*, 2007), GM-CSF is also required to maintain DC populations in peripheral tissues (Greter *et al.*, 2012; Zhan *et al.*, 2012). Thus, we wanted to address whether IFN-I signalling would be required for schistosome-specific Th2 induction by DCs *in vivo*. It was essential to examine whether our findings using BMDCs had relevance to immune activation *in vivo*, as DCs will encounter Ag in a more complex microenvironment, including exposure to bystander Ags and other cell types, which may influence Th2 development. In our adoptive transfer model, *in vitro* generated DCs are exposed to SEA prior to injection, thus their responses will be less influenced by the microenvironment. *In vivo* DCs are exposed to tissue-factors, for example TSLP, TGFβ or IL-10, produced by cells such as epithelial cells and Tregs, which may impact on their phenotype and function. This is particularly the case during patent helminth infection, where the immune system is exposed for prolonged periods to large quantities of Ag, from multiple lifecycle stages, often in the context of tissue damage caused by the parasite. Thus it was worthwhile to assess DC phenotype and the development of Th2 responses in the context of *in vivo* Ag challenge models, but also during patent helminth infection. Moreover, to date, there have been no studies examining the existence of an IFN-I signature in Th2 settings, or any work addressing whether IFN-I impacts on the development of Th2 responses during helminth infection.

We began by characterising the gene expression profile of murine splenic cDCs following i.v. SEA injection, which demonstrated that, like FL-cDCs, these cells displayed an IFN-I signature in response to SEA. Although global expression of IFNAR was not required for SEA-specific Th2 induction in the dLN following subcutaneous challenge with *S. mansoni* eggs, we have demonstrated that optimal Th responses during murine models of helminth infection may depend on IFN-I signalling. We are also investigating whether there is a role for IFN-I in human disease. However, we have so far failed to identify an increase in

systemic IFN-I in *S. mansoni* infected individuals or IFN-I secretion by PBMC-derived human DCs in response to SEA.

5.3.1 SEA-specific IFN-I from mouse splenic DCs, but not human blood-derived DCs

Systemic SEA treatment allowed us to assess whether our findings from FL-cDCs responding *in vitro* were applicable to splenic cDCs responding *in vivo*. We chose to focus on the spleen because previous studies have described the similarities of splenic cDCs to Flt3-L generated BMDCs (Brawand *et al.*, 2002; Naik *et al.*, 2005). However, the primary effector sites for *S. mansoni* infection are the liver and intestines (Wynn *et al.*, 2004), so a model to study the responses of DCs in the liver and liver-draining LNs and intestines and MLN to SEA/eggs would also be of real interest. Although we detected IFN-I production from FLDCs (Chapter 3, Fig. 3.3B), i.v. injection of mice with SEA did not induce elevated IFN-I levels in the serum (data not shown as no detectable IFN-I in any serum samples). There was also no detectable transcript for IFNα1 or IFNβ from splenic cDCs 12h after SEA injection (data not shown as no IFN-I transcripts were detected in any samples). It is possible that upregulation of IFN-I transcripts may occur earlier than the 12h timepoint analysed here. Further, we have yet to measure transcript levels for all the IFN-I subtypes, so it is also possible that other IFN-Is are upregulated at this timepoint. Nevertheless, a range of ISGs was significantly upregulated in splenic cDCs from SEA-injected mice (Fig. 5.1E), indicating the activation of an IFN-I signature. Changes in the expression levels of H2-Ab, CD40 and CD80 mRNA also suggested that SEA may stimulate an activated surface phenotype in splenic cDCs (Fig. 5.1D), and future experiments will assess this comprehensively, using flow cytometry to assess surface phenotype. Together, these data suggest that splenic cDC responses to schistosome egg Ags *in vivo* are similar to those of FL-cDCs *in vitro*, resulting in upregulation of surface molecules associated with phenotypic activation, as well as IFN-I signalling.

The splenic DC data presented here (Fig. 5.1D) is representative of two experiments performed in our clean animal unit. Several subsequent experiments were carried out to address whether the splenic cDC responses are dependent on IFNAR signalling, comparing gene expression profiles in cDCs from WT and *Ifnar1*$^{-/-}$ animals. However, these experiments were carried out in our quarantine facility, which has a lower health status, and PBS-treated animals had higher basal expression levels of the genes analysed

than those seen in our clean facility; consequently no SEA-specific phenotype was discernable in either WT or *Ifnar1*$^{-/-}$ mice (data not shown). For this reason we cannot definitively state that IFNAR is required for the response of splenic cDCs to SEA *in vivo*, and further experiments must be performed to confirm that the WT phenotype is robust. In future experiments, the gene expression profile of splenic DCs following systemic *S. typhimurium* treatment should also be assessed as a Th1 control. This will provide an interesting comparison of ISG induction in splenic DCs in response to a Th1 or Th2 stimulus.

Our DC purification technique requires 5 pooled spleens, which generates around 0.5-1 million cDCs that were sorted directly into Trizol aliquots. These aliquots were kept separate for gene analysis rather than being pooled, providing technical replicates from 5 pooled spleens. These internal replicates indicate that the gene expression data is reliable. However, for more rigorous statistical analysis of such data, multiple repeats must be performed. The statistical power of independent experiments could also be improved by using multiple 5-pooled replicates per condition. We have chosen to sort all cDCs together rather than split the cDC subsets. This was done because the CD8α^{+} subset in the spleen is quite a small proportion of the total cDCs (Shortman and Heath, 2010), and cell numbers were already limiting. However, it is possible that there are gene expression differences between the cDC subsets that could be investigated by scaling up the enrichment and sorting procedure.

As well as characterising the responses of mouse splenic DCs to SEA, we wanted to investigate whether human DCs displayed any of the hallmarks of the IFN-I signature identified in mice following exposure to SEA. In these initial experiments, IFNα was not detectable in any of the supernatants from human DC subsets cultured with SEA (Fig. 5.10B). Of the Flt3-L-generated murine subsets, we have found that the CD8α^{+} cDC-equivalent is uniquely able to produce IFN-I following overnight culture with SEA. Hence, we may have expected to detect IFN-I production from CD141^{+} DCs, the human DC subset that has been shown to most closely resemble murine CD8α^{+} cDCs (Jongbloed *et al.*, 2010; Poulin *et al.*, 2010). This subset, unlike CD1c^{+} cells, expresses TLR3, and secretes IFNβ in response to the TLR3 ligand polyI:C (Hemont *et al.*, 2013; Jongbloed *et al.*, 2010). Jongbloed *et al*. (2010) did not detect IFNα mRNA from CD141^{+} DCs in

response to TLR ligands. However, a recent study of DC subsets in humanised mice demonstrated IFNα production from $CD141^+$ DCs following exposure to dsRNA (MeixIsperger *et al.*, 2013). Thus, this human DC subset, like its murine equivalent, can secrete IFN-I in response to nucleic acid ligands. More analysis is required to establish whether human DC subsets from the blood secrete IFNβ, or whether IFN-I mRNA levels are altered, following exposure to SEA.

Similarly to murine DCs, human DCs express IFNAR on their surface (Severa *et al.*, 2006), and so are able to take up, and are responsive to, IFN-I (Hillyer *et al.*, 2012). Human myeloid DCs, and $CD141^+$ DCs specifically, have been shown to upregulate ISG expression in response to TLR ligands, as well as in response to viral infection (Hillyer *et al.*, 2012; Schulte *et al.*, 2013). Although no secreted IFNα was detectable from human DCs following SEA exposure, it is possible that an ISG expression profile is activated in response to SEA, a possibility that we are actively pursuing. In support of this, the ISGs IFIT2 and IFIT3 have been identified as two genes upregulated by human DCs in response to omega-1 (Bart Everts, personal communication), a strongly Th2-polarising component of SEA (Everts *et al.*, 2009; Steinfelder *et al.*, 2009). We would like to compare and contrast the ISG expression profiles of murine and human DCs following omega-1 and SEA stimulation, to assess the similarities and differences of DC responses to these Ags. Thus, there is still much work to be done to fully characterise the IFN-I signature of human DCs in response to schistosome egg Ags.

5.3.2 Serum IFN-I in mouse and human *S. mansoni* infection

Serum IFN-I levels, particularly IFNα3, were elevated in mice after high dose infection with *S. mansoni*, but not at lower doses (Fig. 5.3-4). Although IL-5 and IL-10 were also upregulated in the serum of the majority of infected animals at a 180 cercariae dose (Fig. 5.4B), there was no correlation between serum IFNα and the levels of these cytokines (Fig. 5.4C). This suggests that an increase in systemic IFNα is not associated with a significant increase or decrease in the Th2 or regulatory response.

All animals in the 180 cercariae group were heavily infected, demonstrated by severe visible liver pathology. However, egg and worm counts from these animals were not performed so we do not yet know whether higher IFN-I production is associated with

greater infection load. Systemic IFN-I was elevated in 180 cercariae infected mice on d49 of infection, however we have yet to examine whether IFN-I levels are significantly above background at other timepoints. Similarly, IFNα/β levels were not above naïve levels on d56 at doses of 20-160 cercariae (Fig. 5.3B). However, this does not rule out the possibility that IFN-I is upregulated at other stages of infection at lower doses. Furthermore, the dose experiments included only 4 mice per dose, due to the variability of systemic cytokine levels this is not enough animals to provide conclusive data. For this reason, it would be informative to determine serum cytokine levels over the course of infection, at different doses. This is a particularly pertinent question as we know that IFNα/β gene expression is upregulated in the liver at specific timepoints of infection: on d42 and d105 following infection with 40 *S. mansoni* cercariae (Fig. 5.5B), thus we may expect to see systemic levels of IFN-I elevated at these timepoints. For this reason, more comprehensive analysis of serum cytokines, with a larger sample size, is required at these timepoints and this dose (40 cercariae) to establish whether systemic IFN-I is also upregulated. However, it is conceivable that systemic IFN-I will not be elevated at these timepoints, despite an increase of IFN-I gene expression, because IFN-I may well be taken up by IFNAR-expressing cells in the tissue site.

The gene expression data in the liver suggests that IFN-I may play a role in immune priming, as well as much later during infection on d105 (Fig. 5.5B). We do not yet know the role of IFN-I at this later timepoint. From d84 onwards the granulomatous response is downregulated in the liver (Wilson *et al.*, 2007), thus it could be speculated that IFN-I is also involved in immune modulation. This could be addressed directly by using a blocking antibody against IFNAR to inhibit IFN-I signalling at this stage (see Section 5.3.4, below). We have also assessed the phenotype of IFNAR-deficient mice on d42 of infection (Fig. 5.6-Fig. 5.8) and have serum samples from these animals so systemic IFN-I levels should be examined in these mice to address the role of IFNAR in this response. We would expect that in the absence of IFNAR, systemic IFN-I would be much reduced.

In these studies, we have not yet addressed the question of what the cellular source(s) of IFN-I is in this setting, or why IFNα/β is only detectable in the serum at higher doses of infection. It is unlikely that a high level of IFN-I systemically can be attributed to DC IFN-I production alone. With a 180 cercariae dose of infection, the egg burden is large, leading

to tissue damage and pathology, primarily in the liver and intestine (Wynn *et al.*, 2004). Tissue damage is likely to release large quantities of nucleic acids from damaged cells, providing ligands for TLRs that initiate IFN-I production. Moreover, it has been shown that DNA damage can induce IFNα production from fibroblasts (Brzostek-Racine *et al.*, 2011), a similar response may be seen from epithelial cells or other cells that are damaged by egg transit. Severe damage to the intestine could also cause a breach of the mucosal barrier, allowing dissemination of bacteria, another source of IFN-activating TLR ligands, such as nucleic acid or LPS. However, if this was the case we would expect to see other cytokines associated with sepsis upregulated in the serum – there was no significant increase in IL-6, IL-12p70 or TNFα in these animals (Fig. 5.4B). All of these factors could potentially contribute to increased IFN-I levels in the bloodstream.

Although IFN-I was elevated in the serum of heavily infected mice, we did not see a similar upregulation in systemic IFNα in humans infected with *S. mansoni*, even in individuals with high faecal egg counts (Fig. 5.11). This is perhaps not surprising, as it has been reported that people with chronic schistosome infection exhibit downmodulation of immune mechanisms (Grogan *et al.*, 1998; Ottesen *et al.*, 1978; Viana *et al.*, 1994). For example, a study of a cohort from a schistosome-endemic area of Zimbabwe indicated that IL-4, IL-10 and IL-13 levels in the serum were lower in *S. haematobium* egg-positive individuals when compared to egg-negative subjects (Milner *et al.*, 2010). Individuals with patent *S. haematobium* infection had higher serum IL-2 and IFNγ levels than those classed egg-negative. Our data from a small sample of individuals from Kenya suggests that circulating IFNα levels have no correlation with infection status (Fig. 5.11). Although we have not measured the levels of other cytokines in the serum samples, members of Prof. David Dunne's lab have done this analysis. We plan to examine whether there is any correlation between IFNα levels and the systemic levels of other cytokines in these samples in due course. Ideally we would have measured these other cytokines ourselves to rule out any variability in the assay process, however the cost of the required reagents was prohibitive.

Systemic IFNα levels were very low in all samples analysed (<5pg/ml), this introduces inherent variability to the dataset as levels are very close to the limit of detection. Further to this, while our IFNα readings are low, they are actually comparable to the median systemic IFNα levels recorded in individuals infected with hepatitis B (HBV) or C (HCV) at

the peak of viremia, which were 5.5pg/ml and 7.2 pg/ml respectively (Stacey *et al.*, 2009). This is compared to a median baseline (level prior to infection) of 3.1pg/ml in HBV infected individuals and 4.7pg/ml in HCV patients (Stacey *et al.*, 2009). Only 40-50% of individuals actually displayed elevation in systemic IFNα during hepatitis infection. This is despite the fact that IFN-I is believed to be essential for host survival in a mouse model of hepatitis (Cervantes-Barragan *et al.*, 2007). Thus, it is not surprising that we saw no significant change in systemic IFNα levels in *S. mansoni* infected individuals.

We have PBMC RNA samples from the same individuals whose serum was tested, so we can next assess ISG expression in human *S. mansoni* infection. This analysis will encompass all cell populations present in the blood and will not solely measure the IFN-I signature from DCs. However, it will provide insights into the level of IFN-I activation in the cells of infected individuals, and a starting point for subsequent studies on sorted specific cell types.

5.3.3 The role of IFNAR in the T cell response to schistosome egg injection

The data from our sole egg injection experiment in *Ifnar1*$^{-/-}$ mice are difficult to interpret at this stage without repeat experiments to compare to. On the face of it, only the Th1 and regulatory aspects of the T cell response were impaired in egg injected IFNAR-deficient animals (Fig. 5.2B). However, the increase in CD4^{+} T cell numbers in the dLNs of *Ifnar1*$^{-/-}$ mice most likely influenced cytokine output. Although *Ifnar1*$^{-/-}$ mice do not have any severe developmental abnormalities (Hwang *et al.*, 1995), this does not rule out alterations to immune cell populations. When cytokine production was determined, taking into account the differences in proportions of CD4^{+} T cells, along with a reduction in IL-10 and IFNγ production, IL-13 levels were also reduced. A more reliable measure of T cell output is required to overcome the difference in T cell numbers. One such method would be to FACS sort CD4^{+} T cells from the dLN, and restimulate these cells with Ag in the presence of irradiated APCs. ICC of dLN T cells would also provide valuable information about the proportion of cytokine-producing cells in the pLN of WT and *Ifnar1*$^{-/-}$ mice. Members of the laboratory are currently in the process of optimising ICC protocols to assess SEA-specific cytokine production, however, the percentage of cytokine^{+} cells in these assays is much lower than can be achieved with a polyclonal stimulus like PMA/Ionomycin. However, we are also now using αCD28/αCD3 as an effective, more physiologically relevant polyclonal

stimulus for ICC. An additional way to assess T cell cytokine output in this setting would be to cross our IFNAR-deficient animals with the KN2 and IL-13-eGFP reporter lines, which would provide information on the levels of IL-4 secretion and IL-13 mRNA expression respectively.

Egg injection into global IFNAR-deficient mice does not directly address the role of DC-specific expression of IFNAR in the induction of the Th2 response against eggs. IFNAR is expressed on lymphocytes as well as myeloid cells (Diamond *et al.*, 2011), so any alteration in the response cannot be directly attributed to an alteration in DC function. To address the importance of IFNAR expression of DCs it is necessary to analyse the T cell response following egg injection into CD11c-cre x IFNAR-flox mice. Chris Engwerda (QIMR, Brisbane, Australia) has these mice and members of his lab are in the process of performing these experiments for us. In our FLDC transfer experiments the SEA-specific Th2 response is absent on d7 following transfer of IFNAR-deficient cells (Fig. 4.6). Our hypothesis is that this inability to prime by transferred IFNAR-deficient FLDCs is due to defective DC migration. If DC migration is affected in global *Ifnar1*$^{-/-}$ mice, in the egg injection model there may well be a defect in Th2 priming at early timepoints after challenge – due to delayed migration – which is rescued by d7. Thus, we would like to compare the kinetics of the developing immune response following egg injection in WT and IFNAR-deficient animals by assessing Ag-specific cytokine production at earlier timepoints after injection.

If our findings with FL-cDCs and GMDCs are applicable *in vivo*, different DC subsets may have a differential requirement for IFNAR. There are a number of different DC subsets that are mobilised from the skin following Ag challenge (Henri *et al.*, 2010). It is likely that dermal DCs depend on Flt3-L for their differentiation (Kingston *et al.*, 2009). However, whether or not GM-CSF is also involved in this process, or is required for dermal DC homeostasis, remains a contentious issue (Edelson *et al.*, 2011; Ginhoux *et al.*, 2009; Greter *et al.*, 2012; King *et al.*, 2010). This means that subcutaneous egg injection is a complex model for identifying the role of DC IFNAR expression in Th2 induction. We do not yet know which DC subsets are key to the induction of Th2 induction against *S. mansoni* eggs into the hind feet, however, depletion of all CD11c^{+} cells ablates the Th2 response following subcutaneous egg injection (Phythian-Adams *et al.*, 2010).

Preliminary data from subcutaneous egg injection in *Batf3*$^{-/-}$ mice (no CD8α^{+} cDCs (Hildner *et al.*, 2008)) suggests that CD8α^{+} cDCs are not required for the induction of the egg-specific Th2 response (Alex Phythian-Adams, Angela Marley). Use of zDC-DTR (DTx depletion of all cDCs expressing Zbtb46 (Meredith *et al.*, 2012)) and pDC-DTR (DTR expression under the control of BDCA2 (Swiecki *et al.*, 2010)) mice will enable us to directly assess the requirement for cDCs and pDCs in egg-specific Th2 induction following subcutaneous egg injection. Th2 induction during patent *S. mansoni* infection will also be assessed in these depletion models. Although we anticipate that findings from the egg injection model will be similar to active infection, there may well be tissue-specific requirements for certain DC subsets. For example, pDCs are found in high numbers in the liver in the steady state (~10% of all haematopoietic cells (Merad and Manz, 2009)) and we know that IFNα mRNA levels are selectively upregulated in the liver at d42 and d105 of *S. mansoni* infection (Fig. 5.5B), suggesting that pDCs may play a role in the induction and regulation of the immune response at this site.

In the spleen, it is known that DCs depend on Flt3-L for their development, and do not require GM-CSF for homeostasis (Kingston *et al.*, 2009; Waskow *et al.*, 2008). As we have identified a similar SEA activation phenotype in FLDCs and splenic cDCs (Fig. 5.1D), it would make sense to build on this initial work and use a systemic route of egg challenge to examine the generation of Ag-specific T cell responses in the spleen of *CD11c-Cre*$^{+/-}$ *Ifnar1*$^{f/f}$ mice, as well as in the pLN following subcutaneous administration of *S. mansoni* eggs. Comparing Th2 induction in these different tissues will further address whether there is a differential requirement for IFNAR expression by DCs resident in lymphoid organs versus peripheral tissues. As previously discussed, the tissues most affected by *S. mansoni* infection include the liver and intestine (Wynn *et al.*, 2004), thus the induction of egg-specific responses in these organs and their dLN should also be assessed in IFNAR-deficient animals. However, during both human and murine *S. mansoni* infection, there are detectable levels of circulating egg Ags in the blood. Thus, the spleen would be continuously exposed to high levels of Ag, if not directly affected by egg traffic, meaning that splenic DCs will also be affected by *S. mansoni* infection.

5.3.4 Helminth infection in IFNAR-deficient mice

A significant reduction in the number of female worms detected in the perfusate of *S. mansoni* infected *Ifnar1*$^{-/-}$ mice may suggest that development of patent infection is inhibited in these animals (Fig. 5.6C). However, the experiment must be repeated to verify this finding, particularly in light of the fact that animals lacking detectable worm pairs did have parasite eggs in the liver and intestine, illustrating the technical variability and limitations of manual perfusion. If the animals in the IFNAR-deficient group that had no detectable female worms are excluded, there is no significant difference in the total worm pairs between groups. For this reason, it is necessary to assess parasitology in follow-up experiments before any conclusions can be made about worm development in IFNAR-deficient mice.

A number of studies have examined the requirement for immune mediators for optimal worm development in the infected host. It is impossible to grow schistosomes in tissue culture, which is highly suggestive that these parasitic worms require cues from the host to develop (Pearce and MacDonald, 2002). Attempts to infect mice that have immune system defects, such as immunosuppressed animals, athymic mice and mice with severe combined immunodeficiency (SCID), demonstrate that worm fecundity is markedly reduced in the absence of a functional immune system (Amiri *et al.*, 1992; Harrison and Doenhoff, 1983). Studies in *Rag*$^{-/-}$ mice (lack T and B cells) suggested that lymphocytes might be key to optimal worm fecundity, further characterisation of worm development in TCRβ-deficient and αCD4-treated animals demonstrated that it is CD4^{+} T cells that provide essential cues to developing worms (Davies *et al.*, 2001). This is in agreement with findings from a previous study in IL-7-deficient mice where worm development was curtailed (Wolowczuk *et al.*, 1999). Initially this result was interpreted as a demonstration that IL-7 acted directly to enhance worm development, however, it was later shown that the requirement for both IL-7 and IL-2 was for optimal CD4^{+} T cell functionality, on which worm development depends (Blank *et al.*, 2006). The nature of the T cell-derived signal has yet to be established. It is known that *S. mansoni* parasites express a receptor that binds human TGFβ (Beall and Pearce, 2001). An early study also suggested that TNFα could rescue worm fecundity in SCID mice (Amiri *et al.*, 1992), however, this was later found not to be an essential factor (Cheever *et al.*, 1999). It is reasonable to suggest that if IFNAR signalling is required for optimal CD4^{+} T cell function, IFNAR-deficiency may impact

on worm development; however, this requires further work to establish whether there is a phenotype in these mice.

Although *Ifnar1*$^{-/-}$ animals have no obvious developmental defects, there do appear to be some differences in the immune system of these animals compared to WT mice, with a significant increase in the size of the T cell compartment and ~25% more CD4^{+} T cells in the pLN, as well as a significant reduction in the cellularity of the MLN in naïve mice. An alternative way to investigate the role of IFNAR in helminth infection would be to administer the IFNAR1-blocking antibody, MAR1-5A3 (Sheehan *et al.*, 2006), to WT mice. An advantage of this method is that the role of IFNAR could be assessed at different stages of *S. mansoni* infection by antibody administration at different timepoints, for example at the early stages of Th2 priming (d28-42) and in chronic infection (d84+). The effectiveness of this approach has been demonstrated in West Nile virus infection (Pinto *et al.*, 2011). However, in this setting the researchers administered the antibody only once to block IFNAR function for 5 days. Thus we would need to assess whether the antibody could be given multiple times over a longer period during *S. mansoni* infection. The disadvantage of a blocking antibody is that the efficacy of treatment may not be comparable across all tissues. Although MAR1-5A3 could provide a good model to investigate the role of IFNAR, the most relevant experiment at this stage would be *S. mansoni* infection in *CD11c-Cre*$^{+/-}$*Ifnar1*$^{f/f}$ mice to establish the importance of DC expression of this receptor. However, we do not currently have this mouse strain in order to perform these studies.

In the liver of *S. mansoni* infected globally IFNAR-deficient mice, only the development of the IL-10 response was significantly reduced (Fig. 5.7B). A role for IFN-I and IFNAR in the induction of IL-10 has been identified in a variety of settings. For example, IFNAR is required for IL-10 production in the lung during influenza infection (Arimori *et al.*, 2013). It has also been demonstrated previously that IFN-I is required for IL-10 induction in the liver specifically. In IFNAR-deficient mice immunised with a DNA-based vaccine, inflammatory CD8^{+} T cell responses were increased in the liver (Dikopoulos *et al.*, 2005). However, hepatic Treg numbers were not altered in these animals. Instead, IFN-I was required for the induction of IL-10 production from CD4^{+} Foxp3^{-} T cells. Similarly, we identified no deficit in Treg numbers in the livers of *Ifnar1*$^{-/-}$ mice during *S. mansoni* infection,

suggesting that a comparable mechanism may be required for IL-10 induction in this tissue during schistosome infection.

Facets of the MLN T cell response were reduced in both *S. mansoni* and *H. polygyrus* infection of IFNAR-deficient animals (Fig. 5.7C and Fig. 5.9D), whilst the induction of the Th2 response was unaffected in the liver of *S. mansoni* infected mice (Fig. 5.7B). A selective impairment in LN responses may reflect our finding that Flt3-L dependent BMDCs are uniquely dependent on IFNAR for their function. If GM-CSF is, as reported, required for the homeostasis of DCs in peripheral tissues (Greter *et al.*, 2012), and tissue-resident DCs share the characteristics of GMDCs, these cells may be able to maintain T cell functionality in peripheral sites during helminth infection in the absence of IFNAR. To investigate this further, a comprehensive analysis of the Th2 response in the spleen, liver dLN and intestines of IFNAR-deficient mice during *S. mansoni* infection is required. The lifecycle stages of *H. polygyrus* that occur within the rodent are restricted entirely to the gastrointestinal tract of the host (Maizels *et al.*, 2012a), thus examination of the immune response in the lamina propria during *H. polygyrus* infection will demonstrate whether or not T cell responses are also ablated in the tissue, as well as the MLN, of IFNAR-deficient mice.

The possibility of a cell-intrinsic defect in T cell responses cannot be ruled out in helminth infection of global *Ifnar1*$^{-/-}$ mice. As discussed in the introduction to this chapter, IFN-I can directly influence T effector functions, and has also been shown to promote T cell survival, at least *in vitro* (Marrack *et al.*, 1999). In 2 out of 3 *H. polygyrus* infected *Ifnar1*$^{-/-}$ mice, IL-10 responses were above WT levels (Fig. 5.9D). Clearly, this result was not significant and the IL-10 response must be assessed further in future experiments. An increase in the regulatory response may be the cause of limited IL-4, IL-5 and IFNγ production seen in these 2 mice (Fig. 5.9D). Treg numbers are elevated in the MLN by d6-7 of *H. polygyrus* infection (Finney *et al.*, 2007; Rausch *et al.*, 2008), and this cell population plays a central role in controlling the effector response (Grainger *et al.*, 2010). The proportion of Treg cells in the MLN of WT and IFNAR-deficient *H. polygyrus* mice should be assessed. *In vitro* studies have found that IFNα can inhibit Treg differentiation and function, both by acting on T cells directly and by altering DC activity (Golding *et al.*, 2010; Pace *et al.*, 2010). Thus, Treg function may be augmented in *Ifnar1*$^{-/-}$ mice infected with *H. polygyrus*, inhibiting T

effector responses. IL-10 production (Fig. 5.7B-C) and Treg numbers (Fig. 5.8B-C) were not increased in *S. mansoni* infected IFNAR-deficient mice, thus increased regulation does not likely account for the reduction in Th responses in this setting.

The work detailed in this chapter has shown definitively that there are impairments in the induction of adaptive responses against helminths in the absence of IFN-I signalling. Whether or not this is due to DC dysfunction remains to be determined. The existence or otherwise of an IFN-I signature in human responses to helminths is an exciting new area of investigation for us. However, there is still much work to be done in this area, and in the *in vivo* mouse studies. Irrespective of this, the data we have generated now provides a solid platform for future studies aiming to further clarify these issues.

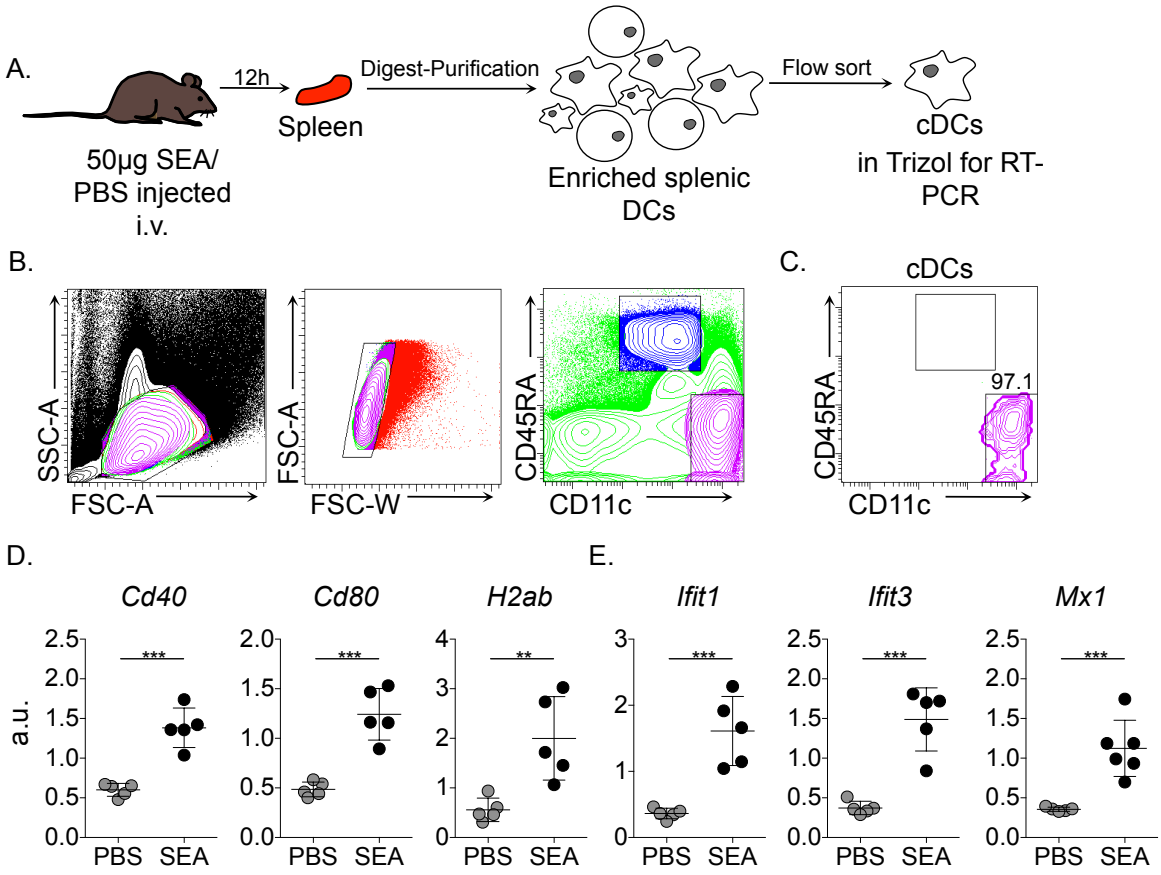


Figure 5.1 Systemic SEA administration induces gene expression changes in splenic cDCs. Mice were injected intravenously with SEA or PBS, spleens were harvested 12h later and processed to enrich for DC subsets. Splenic cDCs were FACS sorted from enriched cells (B-C) and RT-PCR analysis of activation markers (D) and ISGs (E) performed on mRNA purified from sorted cells. Gene expression normalised to *Gapdh* expression. Data representative of 2 experiments, spleens from 5 animals pooled per group. **P<0.01, ***P<0.001

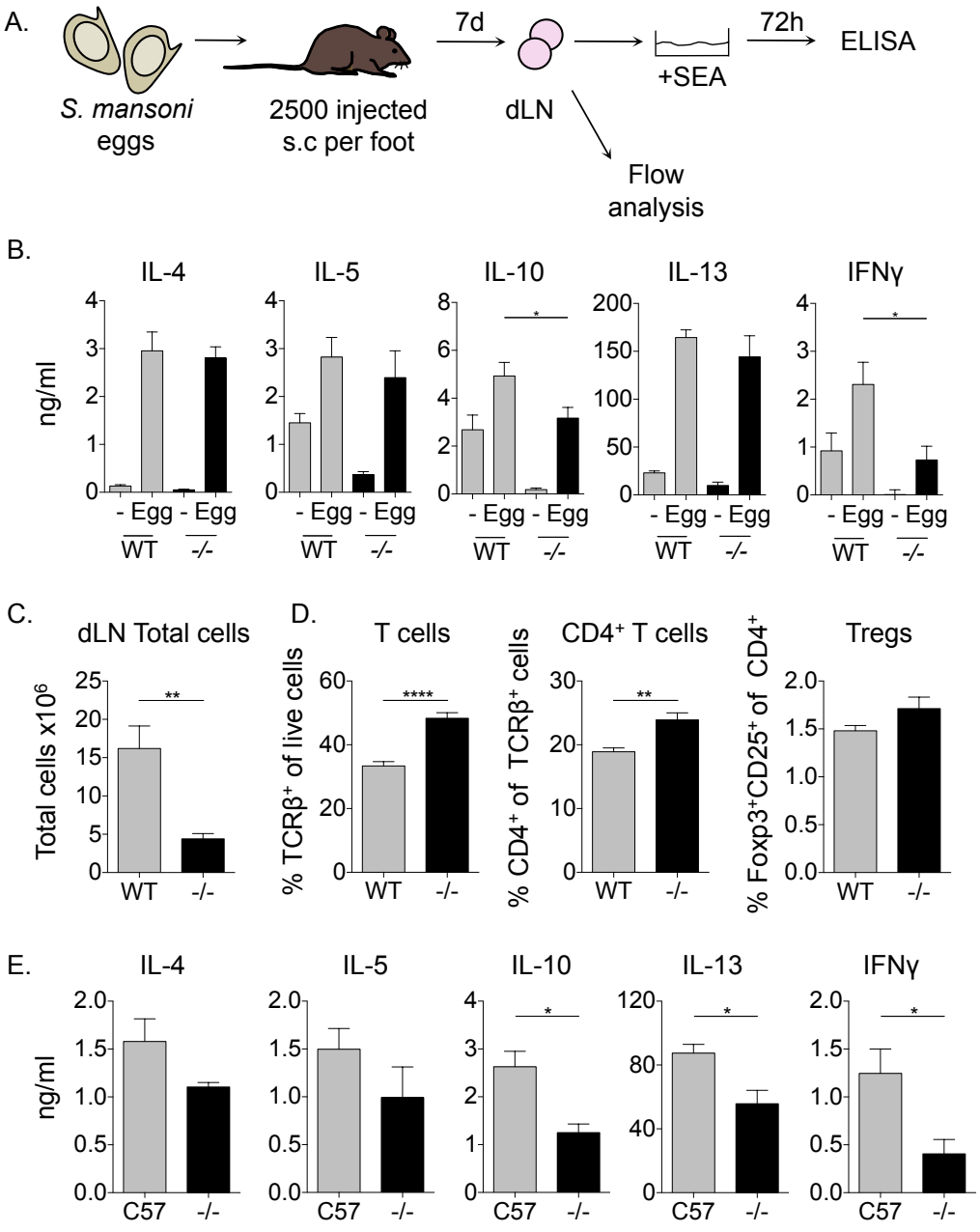


Figure 5.2 There is no significant impairment in Th2 induction in the draining lymph node of *Ifnar1*$^{-/-}$ following *S. mansoni* egg injection.
WT or *Ifnar1*$^{-/-}$ animals received 2500 eggs into the top of each hind foot. On d7 following egg injection, the draining LN (Egg) were harvested, and the mesenteric LN (-) also harvested as a distal control. LN cells were counted (C), restimulated with SEA (B) or prepared for flow analysis (D). ELISAs were performed on cell supernatants after restimulation to assess the Ag-specific recall response (B). The proportion of T cells was calculated as the percentage of live-singlet cells that were TCRβ$^+$, the proportion of CD4$^+$ T cells as the percentage of TCRβ$^+$ live-singlets that were CD4$^+$, Treg percentages represent the proportion of CD4$^+$ T cells that were also Foxp3$^+$ CD25$^+$ (D). Concentrations of T cell cytokines were calculated per 0.1 x10^6 CD4$^+$ T cells (E). Data from 1 experiment, representative of 6 animals per group. *P<0.05, **P<0.01, ****P<0.0001.

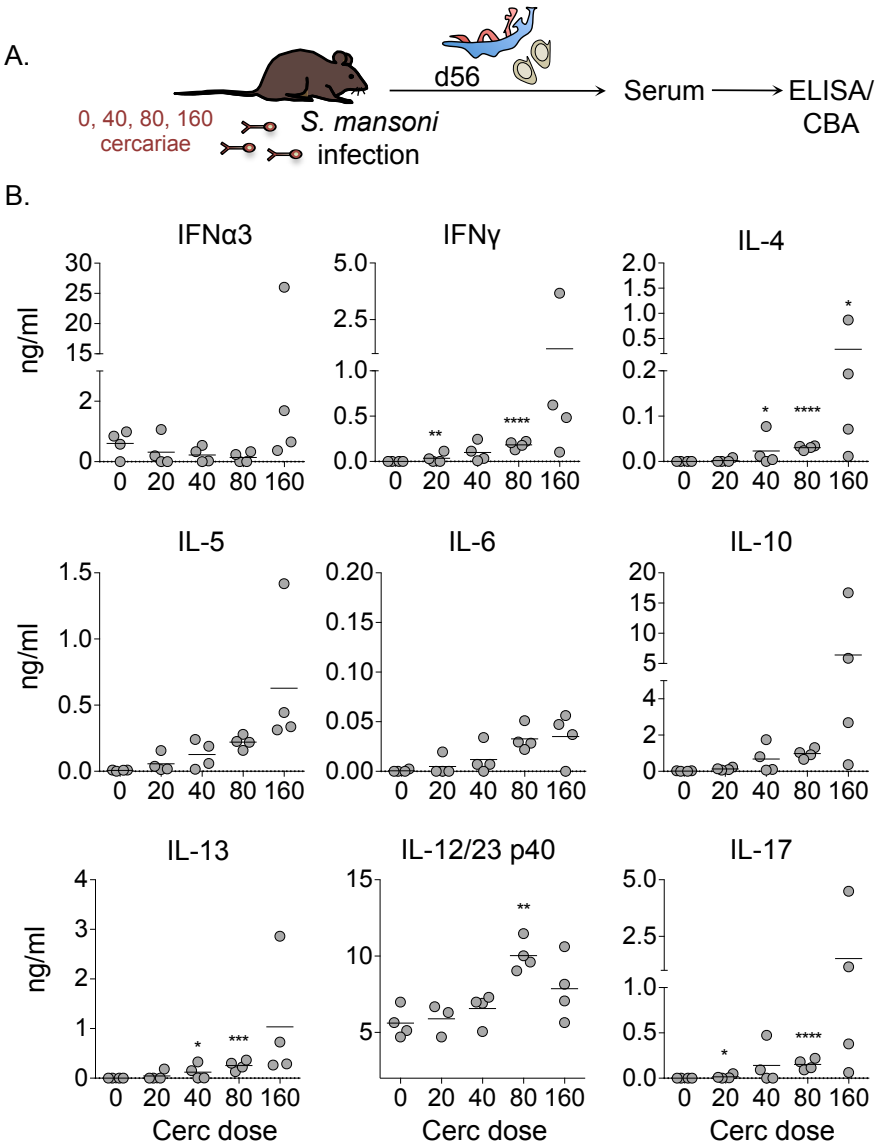


Figure 5.3 Th2 and inflammatory cytokines are significantly elevated on d56 of infection in the serum of animals infected with an 80 cercariae dose.
Animals were infected with *S. mansoni* cercariae and serum samples taken at d56 of infection for assessment of cytokine levels by ELISA/ CBA (B). Data from 1 experiment, 4 mice per group. *P<0.05, **P<0.01, ****P<0.0001. Significance in B compared to cytokine level at 0 cerc dose, data was logarithmically transformed prior to statistical analysis.

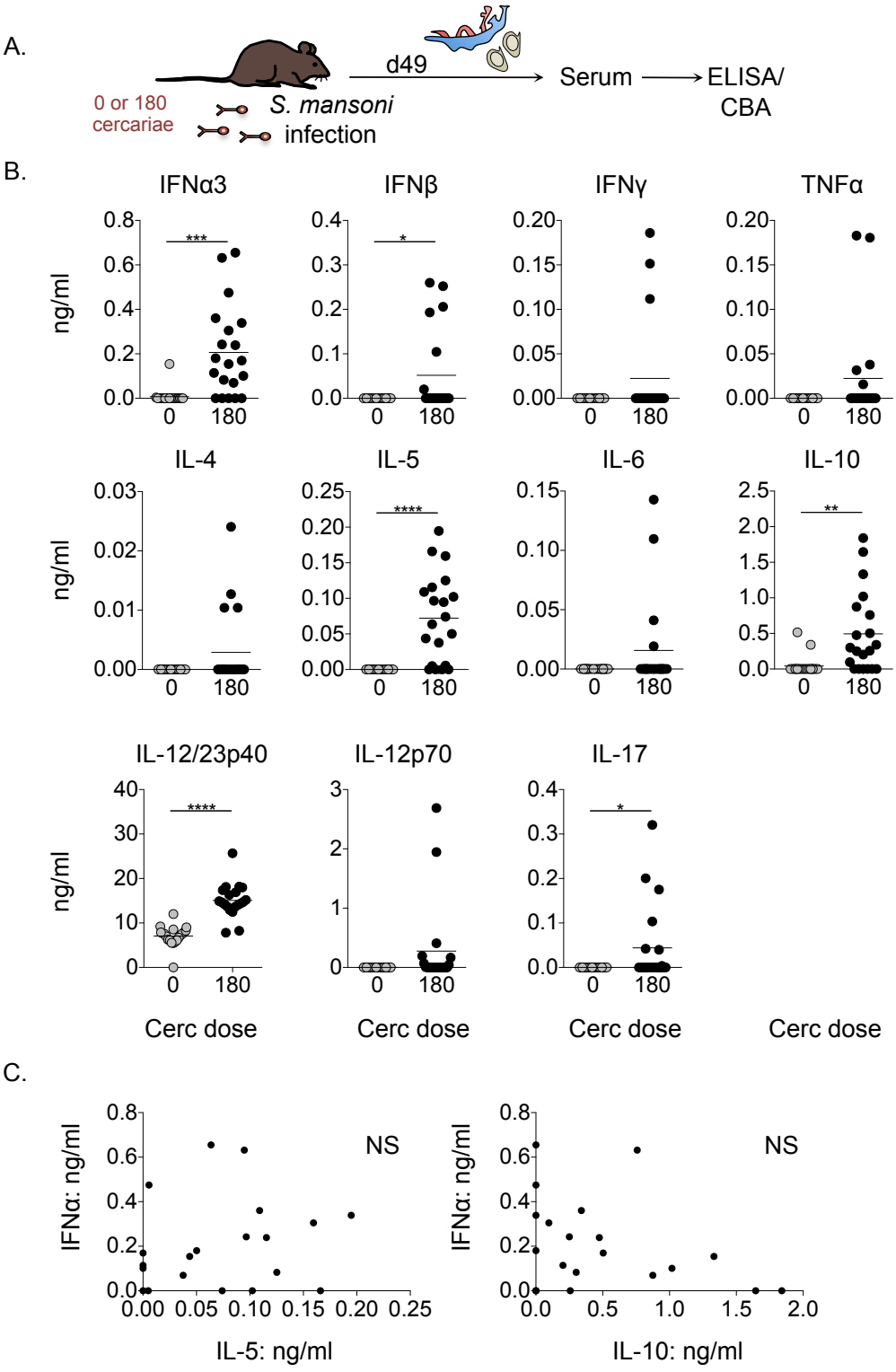


Figure 5.4 IFN-I levels are raised on d49 of infection in the serum of animals infected with 180 cercariae.
Animals were infected with 180 *S. mansoni* cercariae and serum samples taken at d49 of infection for assessment of cytokine levels by ELISA/CBA (B). IFNα levels in the serum of infected mice were compared to IL-5/ IL-10 levels present in these samples (C). No IL-13 was detected. 20 mice per group, data combined from 2 experiments. *P<0.05, **P<0.01, ***<0.001, ****P<0.0001. NS = No significant correlation. Correlations were calculated using Pearson r test on parametric data, and Spearman r tests on non-parametric data.

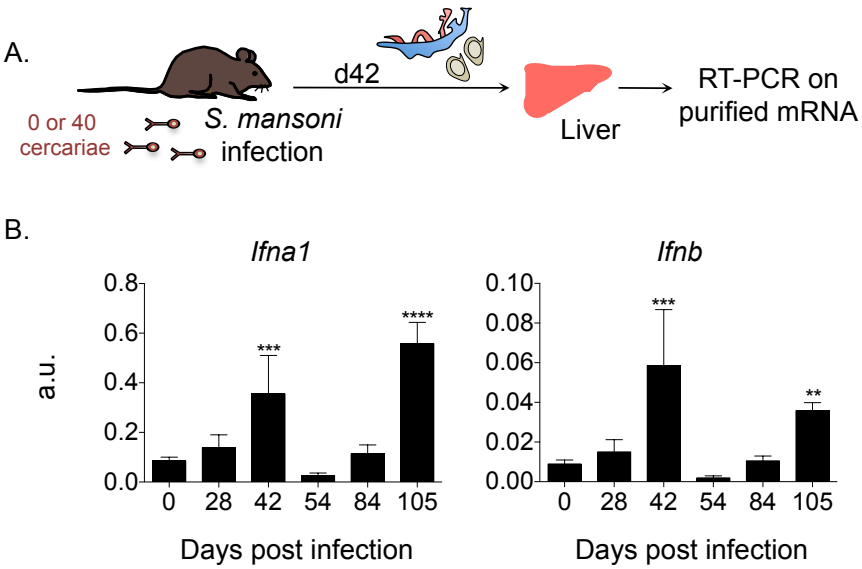


Figure 5.5 IFN-l mRNA levels are elevated on d42 and d105 of infection.
Animals were infected with 40 *S. mansoni* cercariae and killed on d28, 42, 56, 84 or 105. Liver samples were collected and RT-PCR analysis performed on purified mRNA (B). Expression of gene of interest compared to the housekeeping gene, ubiquitin. Data combined from 2 experiments. Statistical analysis compares gene level compared to naïve mice (0). **P<0.01, ***<0.001, ****P<0.0001.

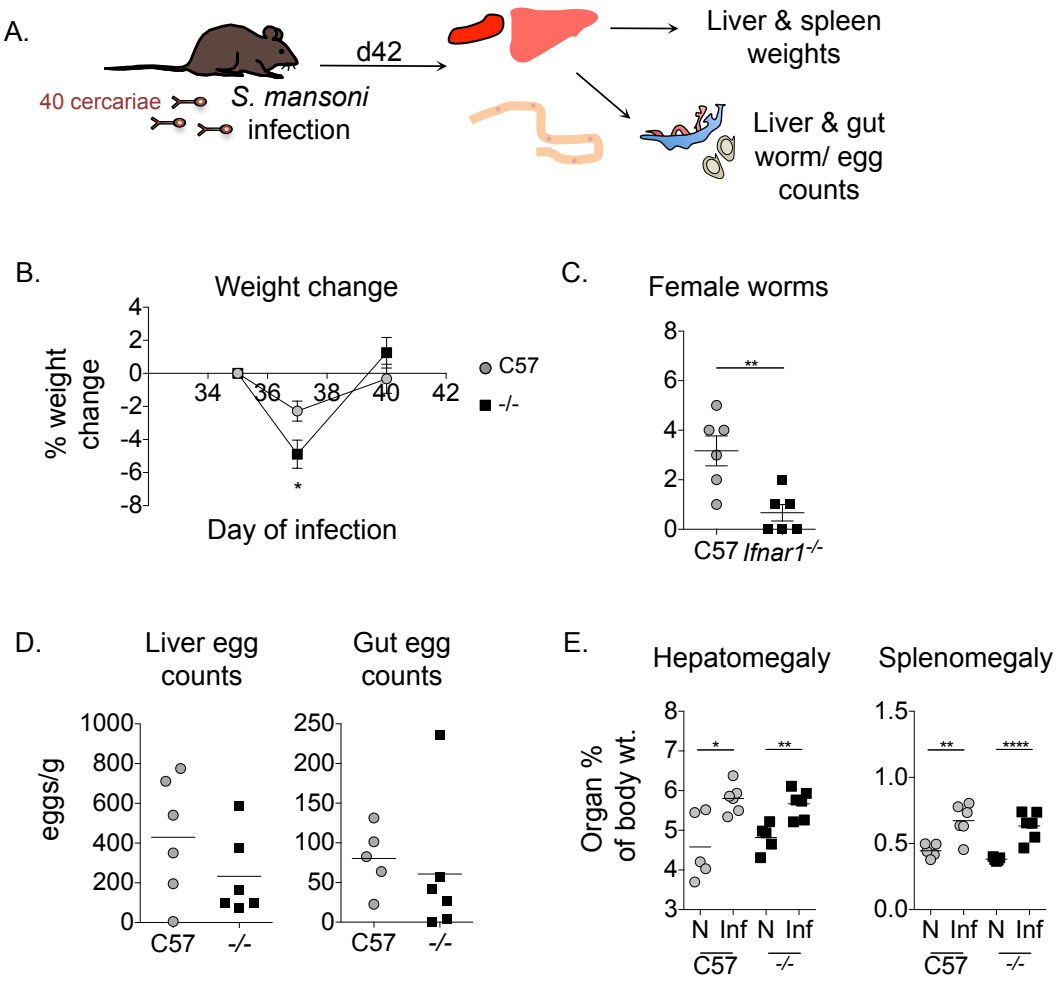


Figure 5.6 *Ifnar1*$^{-/-}$ *S. mansoni*-infected animals have reduced worm pairs on d42 of infection.
Mice were infected with 40 cercs and weighed from d35 onwards (B). On d42 of infection, animals were sacrificed, weighed and heart perfusions performed. Spleens and livers were harvested and weighed. Female worms present in perfusate were counted. Egg counts were performed on liver and gut samples following overnight digestion (D). Animal and tissue weights were used to calculate hepato- and splenomegaly (E). Data from 1 experiment, 6 mice per group. *P<0.05, **P<0.01, ****P<0.0001.

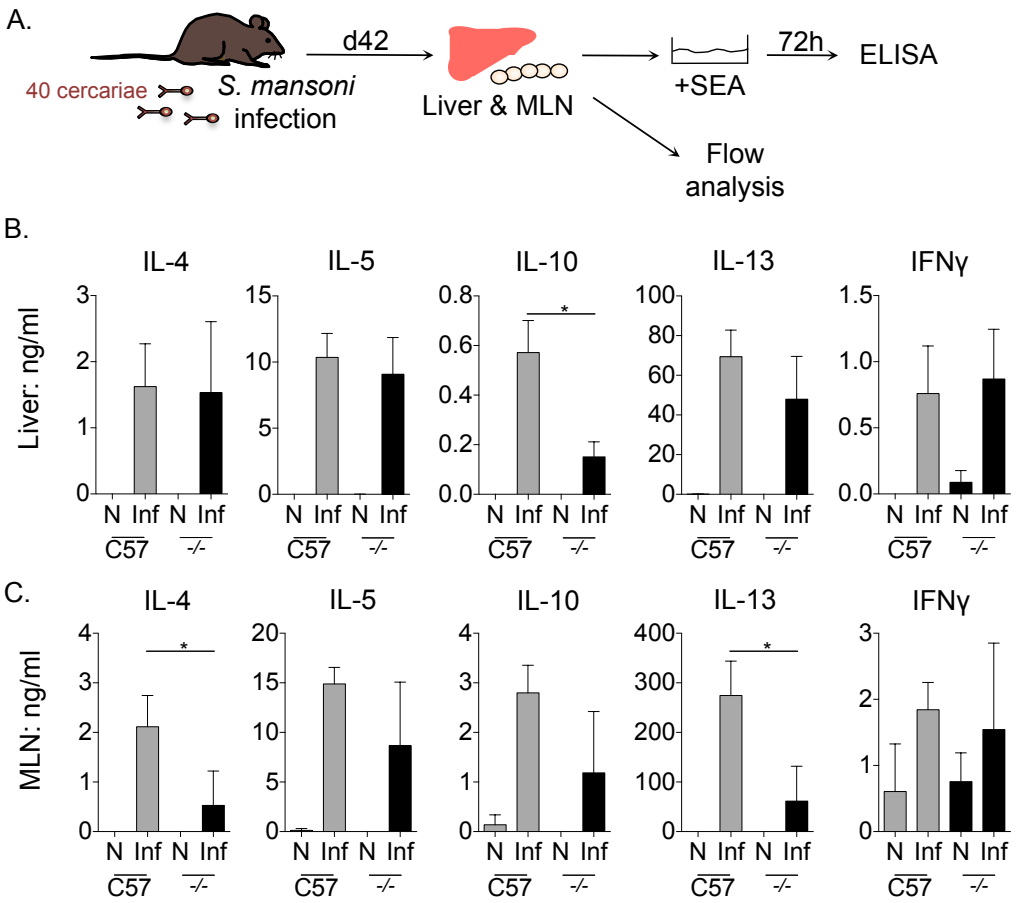


Figure 5.7 *Ifnar1*$^{-/-}$ *S. mansoni*-infected animals display defective Th2 responses in the MLN on d42 of infection.
Mice were infected with 40 *S. mansoni* cercariae and on d42 of infection, livers and MLNs were harvested. Liver (B) and MLN (C) cells were then restimulated with Ag and supernatants collected for ELISA. Medium background subtracted (B-C). Data from 1 experiment, 2 naïve livers pooled per replicate, giving 2 naïve replicates, 4-5 individually prepared infected livers per group. MLN data represents 4-5 individual mice per group. *P<0.05.

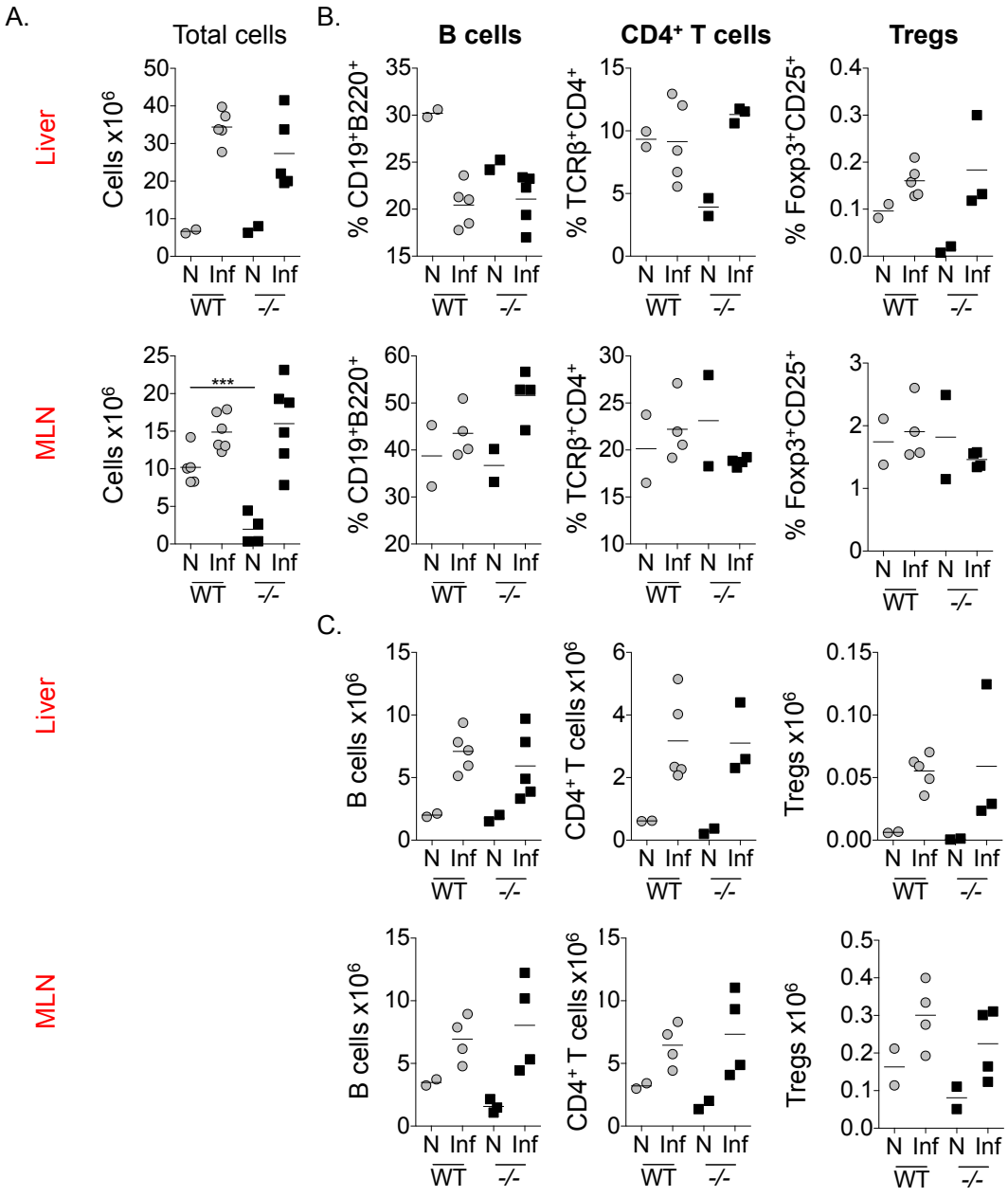


Figure 5.8 WT and *Ifnar1*$^{-/-}$ *S. mansoni*-infected animals have comparable proportions and actual numbers of immune cell populations in the liver and MLN on d42 of infection.

Livers and MLNs were harvested, cells counted (A) and cells were stained for analysis of cell populations by flow cytometry. Proportions of cell populations were calculated as a percentage of intact live-singlets (B). Actual numbers of cell populations was calculated from cell counts using percentages from flow analysis (C). Data from 1 experiment. ***P<0.001.

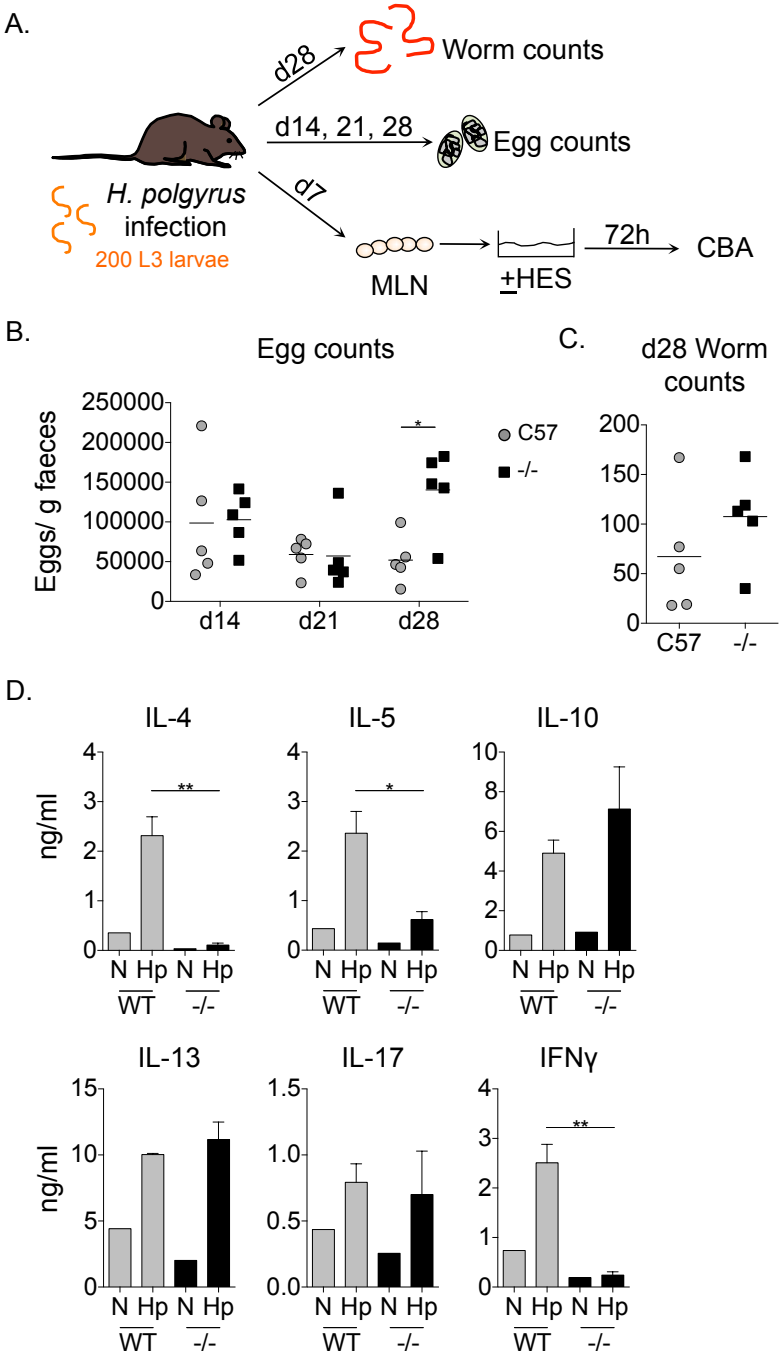


Figure 5.9 *Ifnar1*$^{-/-}$ mice infected with *H. polygyrus*, show increased egg burden at d28 of infection and a reduction in T cell cytokines in the MLN on d7.

Animals were infected with L3 *H. polygyrus* larvae, at d14, d21 and d28 faeces were collected and egg counts performed (B). On d28 worms still within the small intestine were counted (C). On a separate set of animals, MLN were harvested and restimulated with Ag on d7 of infection. CBAs were performed on cell supernatants to assess cytokine production (D). Medium background subtracted in D. Data from 1 experiment, representative of 2 animals pooled (naïve groups) and 3 individual animals (infected groups). *P<0.05, **P<0.01.

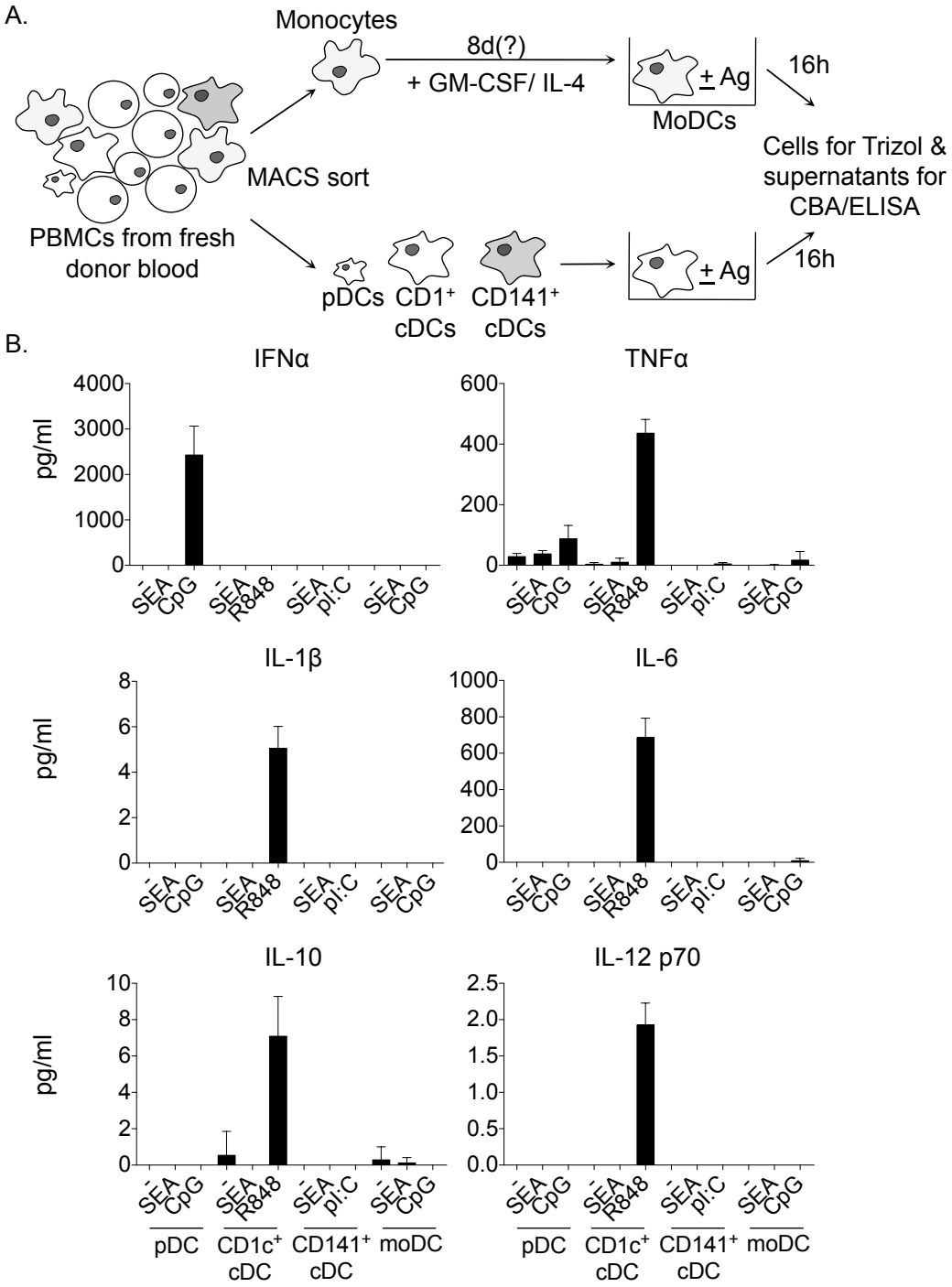
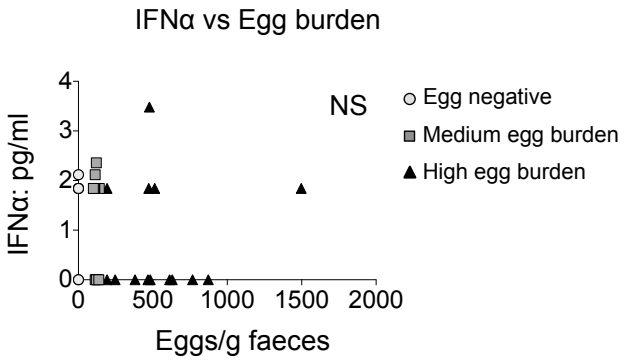


Figure 5.10 IFNα is not secreted in response to overnight SEA stimulation by DC subsets derived from the PBMCs of Western donors.
DC subsets and monocytes were MACS sorted from PBMCs, following purification of cells from whole blood. Monocytes were cultured for 8d with GM-CSF and IL-4 to generate moDC. Directly purified DCs or moDCs from the same donor were cultured overnight with or without Ag plus growth-factors (see Methods), supernatants for ELISA/CBA were collected (B). Data from 3 experiments pooled, 1-2 wells per stimulation.

A.



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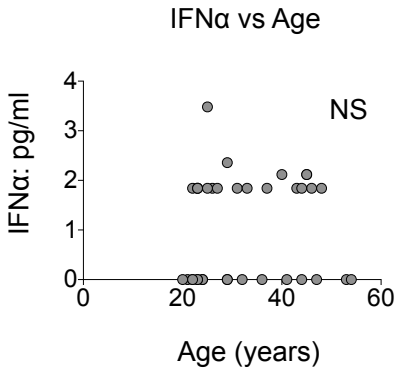

Figure 5.11 Serum levels of IFNα did not correlate with *S. mansoni* faecal egg burden or age.
Median ages for egg negative, medium egg burden and high egg burden individuals were 36.5, 30.5 and 25 years respectively. Sample size of 10 people per group, from a cohort in an endemic area of Kenya (courtesy of David Dunne). NS = No significant correlation. Correlations were calculated using Spearman's r test.

6.0 SCHISTOSOME-SPECIFIC IMMUNE ACTIVATION IN THE SMALL INTESTINE

6.1 Introduction

During *S. mansoni* infection, Th2 activation is required for successful transit of parasite eggs out of the host, as well as for protection of the host from a dangerously Th1-skewed response (Brunet *et al.*, 1997; Fallon *et al.*, 2000; Jankovic *et al.*, 1999). However, a balanced response to *S. mansoni* is essential, as excessive Th2 leads to severe fibrosis and increased mortality (Hoffmann *et al.*, 2000). In the absence of IL-4, impaired egg migration results in their accumulation in the intestinal wall, leading to excessive inflammation at this site (Brunet *et al.*, 1997; Fallon *et al.*, 2000). A failure to control intestinal damage also likely leads to the breakdown of barrier function and translocation of bacteria in *S. mansoni* infected IL-4-deficient mice, leading to higher levels of systemic LPS and inflammatory mediators (Fallon *et al.*, 2000). Thus, the generation of a functional Th2 response and the subsequent control of egg-induced pathology in the intestine is integral to host survival, but must also be regulated to avoid severe immune-mediated fibrosis.

Although our lab has shown that DCs are required for induction of the Th2 response in the spleen and liver during *S. mansoni* infection, and the popliteal LN following *S. mansoni* egg injection (Phythian-Adams *et al.*, 2010), the role of DCs in the intestinal immune response has not yet been assessed. Th2 immunity is critically involved in the development of granulomas around parasite eggs, protecting the host against toxic egg products and egg-mediated intestinal damage. However, excessive Th2 is also damaging, thus there are both regulatory and Th1 facets to the egg-specific response during *S. mansoni* infection (Herbert *et al.*, 2008; Hoffmann *et al.*, 2000; Phythian-Adams *et al.*, 2010). It is our hypothesis that DCs localised in the tissues that are directly affected by egg traffic – the liver and the intestine – play a vital role in orchestrating the immune response during *S. mansoni* infection.

Studies have identified a role for $CD103^+$ $CD11b^+$ LP DCs and $CX3CR1^+$ LP MΦs in the generation of Tregs in the steady state (Coombes *et al.*, 2007; Hadis *et al.*, 2011), whilst $CD103^+$ $CD11b^+$ LP DCs are also involved in Th17 generation and protective responses

against pathogenic bacteria (Persson *et al.*, 2013; Satpathy *et al.*, 2013). However, how intestinal DCs and MΦs respond during helminth infection is currently poorly defined, as is which specific DC subsets are involved in activating the immune response at this site. Experiments in this chapter use flow cytometry to characterise the myeloid populations in the LP during patent *S. mansoni* infection, with a view to future work that will investigate the functional role of these cells in priming or regulating Th2 inflammation in the intestine.

In these studies we have focused on the small intestine, this was primarily to minimise the workload required for processing samples to ensure optimal cell survival. Both the large and small intestine have heavy egg burden during *S. mansoni* infection (Saoud, 1965; Tiboldi, 1979), and exhibit a granulomatous Th2 response during *S. mansoni* infection, downregulation of this response occurs at the chronic stage of infection in the colon, but not the small intestine (Turner *et al.*, 2011; Weinstock and Boros, 1981).

One of the long-term aims of the lab is to understand the role of the different gut-associated DC subsets in the initiation of the egg-induced Th2 response. Whilst determining the phenotype of DCs in the intestinal tissues and the MLNs during *S. mansoni* infection was one approach we used to address this goal, we also aimed to develop a more controlled and reductionist model of synchronous egg exposure to complement the more complex active infection system. Such a model would allow analysis of DC phenotype at the earliest possible points following egg exposure, enabling identification of the specific DC subsets that initiate the egg-specific response in the intestines and MLNs. This analysis is more difficult in patent infection because it is impossible to know exactly when egg deposition begins and so precisely when DCs will first be exposed to and activated by egg Ags.

Because of these potential complications with examining DC responses in actively infected mice, we developed a surgical model of synchronous egg delivery subserosally, directly into the intestinal tissue of non-infected animals. CD11c-depletable mice were utilised to directly address whether any egg-specific immune activation was dependent on $CD11c^+$ cells, thus beginning to unravel the importance of DCs in the generation of egg-specific responses in a mucosal tissue site.

Following initial exposure to eggs, we hypothesise that the initiation of type 2 immunity requires DC migration from the intestinal tissue to the dLN. Although beyond the scope of this PhD, future experiments will use a surgical lymphatic cannulation approach to investigate this. This approach will enable characterisation of the cells migrating in intestinal lymph in response to either patent *S. mansoni* infection or subserosal egg injection. Lymphatic cannulation studies have generally only been carried out in veterinary studies of large animals (Pullinger *et al.*, 2007; Thornbury *et al.*, 1993) or rats (Cerovic *et al.*, 2009; Milling *et al.*, 2006; Pugh *et al.*, 1983). However, a small number of groups have developed these techniques to allow micro-surgical cannulation of laboratory mice. Although it is possible to cannulate the intestinal lymph vessels, this approach provides only miniscule volumes of lymph for analysis (~0.2-0.4µl per mouse) (Schulz *et al.*, 2009). As an alternative approach, following removal of the mesenteric lymph nodes, the thoracic lymph duct can be cannulated (Milling *et al.*, 2010), yielding large numbers of DCs that are clearly migrating from the intestine, as these cells are not present in the lymph of animals that have not undergone mesenteric lymphadenectomy (Cerovic *et al.*, 2012).

The ability to collect large numbers of cells from the intestinal lymph of egg-treated or *S. mansoni* infected animals would allow us to go beyond characterisation of migrating DCs, to perform functional studies of these cells. However, to be able to carry out this approach during *S. mansoni* infection in future studies, we first needed to address whether mesenteric lymphadenectomy would be prohibitive to parasite development or to the generation of a functional immune response. For this reason, we characterised acute *S. mansoni* infection in mice lacking MLN, as detailed in this chapter.

Thus, this chapter details a range of experiments designed to develop innovative new approaches to directly address the role of DCs in Th2 orchestration in the intestines and MLNs – relevant sites during *S. mansoni* infection. This chapter also provides the first insights into the phenotype of the intestinal DC compartment during *S. mansoni* infection. The egg injection model we have developed provides a relevant tissue in which to study the responses of Flt3-L dependent DC subsets to egg challenge *in vivo*. Furthermore, it will allow us to investigate whether IFN-I signalling is required for the induction of egg-specific Th2 responses in a tissue that is directly affected by egg transit during *S. mansoni* infection.

6.2 Results

6.2.1 Myeloid cell populations in the small intestine of *S. mansoni* infected mice

The DC populations resident in the LP of the steady state small intestine have been well characterised (Cerovic *et al.*, 2012; Persson *et al.*, 2013; Schulz *et al.*, 2009; Varol *et al.*, 2010). Before investigating the phenotype and function of DC populations in response to schistosomes, we first sought to confirm we could replicate these results in naïve mice. Analysis was restricted to $CD45^+$ cells (all haematopoietic cells, excluding erythrocytes and plasma cells) in order to remove any non-immune cell populations; autofluorescent $SiglecF^+$ eosinophils were gated out as they express CD11b and other myeloid markers (Fig. 6.1A) (Bain *et al.*, 2012). $Gr\text{-}1^+$ (Gr-1 antibody recognises cells expressing Ly6C/G) cells were also gated out to exclude neutrophils and inflammatory monocytes. DC populations could be identified as $F4/80^-$ $CD11c^+$ MHC II^+ cells; the different DC subsets were delineated by their expression of CD11b and CD103 to give four defined subsets (Cerovic *et al.*, 2012)(Fig. 6.1A). $F4/80^+$ MHC II^+ MΦs also expressed CD11c and high levels of the fractalkine receptor, CX3CR1 (Fig. 6.1A-B), in agreement with published data (Niess *et al.*, 2005), and were $CD103^-$ (Fig. 6.1D). All of the $CD11c^+$ MHC II^+ populations in the LP expressed some level of the co-stimulatory molecules CD80 and CD86 in the basal state (Fig. 6.1E-F). Although LP MΦ are known for their high level expression of CX3CR1, the DC subsets present in the LP also expressed this marker to some degree, when compared to cells from a CX3CR1-$eGFP^-$ mouse (grey shaded histogram, Fig. 6.1D). The findings from these characterisation experiments demonstrated that our cell isolation techniques and staining protocols were in agreement with previous studies in naïve mice.

Prior to investigating the functional role of myeloid cells in the small intestine during patent *S. mansoni* infection, we first characterised the populations present at different timepoints of infection (Fig. 6.2B). On d42, approximately 1-2 weeks after the commencement of egg deposition (Pearce and MacDonald, 2002), there was no discernible change in the total cell numbers recovered from the LP of infected animals, compared to naïve (Fig. 6.2C). The myeloid compartment in the small intestinal LP of infected mice was also comparable to naïve mice; there were similar numbers of DCs and MΦs in the LP (Fig. 6.2E), and no discernible influx of $Ly6C^{hi}$ $CD11b^+$ inflammatory cells (Fig. 6.2D). The proportions of the different LP DCs subsets, delineated by their expression of CD11b and CD103, were unaltered on d42 of infection, compared to naïve animals (Fig. 6.2F).

There was also no discernible change in total cell numbers in the LP on d56 of *S. mansoni* infection (Fig. 6.3B). However, on d56 of infection, by which time the intestine is severely affected by egg-induced pathology (Turner *et al.*, 2012), there were discernible changes in the myeloid population. At this stage, there was an influx of $Ly6C^{hi}$ $CD11b^{+}$ inflammatory monocytes into the small intestine (Fig. 6.3C), however this was variable between animals. The inflammatory monocyte population were uniformly $CD103^{-}$, but expressed variable levels of MHC II and F4/80 (Fig. 6.3G).

The percentage of LP MΦs in each sample was calculated as the proportion of all live intact-singlets that were $Ly6C^{-}$ MHC II^{+} $F4/80^{+}$ (using event counts from FACS data). To quantify the total number of MΦs in the SI LP, the number of viable leukocytes was multiplied by the percentage of MΦs as determined by flow cytometry. This technique is used throughout to calculate the percentage and total numbers of cell populations in infected tissues. Due to the low number of naïve samples in these experiments, it is not possible to perform reliable statistical analysis on the changes to the LP myeloid populations during infection. However, there was a marked decrease in the percentage of LP MΦ on d56 of infection, and in the absolute number of LP-resident MΦs in infected animals (Fig. 6.3C-D). A minor increase in the percentage of $F4/80^{-}$ $CD11c^{+}$ MHC II^{+} DCs did not translate to a discernible alteration in this population when extrapolated to actual cell numbers (Fig. 6.3C-D).

Of the different LP DC subsets, the only population that displayed marked change in absolute cell numbers was $CD103^{+}$ $CD11b^{+}$ DCs, with a dramatic reduction in this population on d56 of infection (Fig. 6.3E-F). This indicates that increasing egg burden in the intestine of *S. mansoni* infected mice (Turner *et al.*, 2012) has marked effects on the myeloid cell populations in the small intestine, but does not affect all populations equally.

On d42 of infection, there was no discernible change in the surface phenotype of LP DC subsets or LP MΦ, illustrated by comparable expression of CD40 by these cells from naïve and *S. mansoni* infected animals (Fig. 6.4A). However, there was a clear reduction in the expression of CD40 and CD86 on all DCs, as well as LP-resident MΦs, on d56 of infection (Fig. 6.4B-C). In particular, expression of CD40 on $CD103^{-}$ $CD11b^{-}$ LP DCs was markedly

reduced at this timepoint (Fig. 6.4B). It is clear that at the early stages of infection the myeloid population in the small intestine is largely unaltered by initial egg deposition. However, as infection progresses and egg-induced pathology becomes more severe, there are stark changes in the make-up of the myeloid compartment, including an inflammatory cell influx and down-regulation of activation markers on LP-resident populations.

6.2.2 Subserosal egg injection: A model of synchronous egg delivery

Oral administration of Ag is a relatively straight-forward way of challenging the intestinal immune system, but is also a route that can induce oral tolerance (Mowat, 2005). As an initial approach to try to generate a more controlled mucosal Th2 response to schistosome eggs than active infection, we administered mice with schistosome eggs or varying doses of SEA by oral gavage and measured the induction of T cell responses in the MLN 7d later (Fig. 6.5A). Very little Ag-specific T cell cytokine production was detectable at this timepoint; no IL-5 or IL-13 was detectable, whereas IL-4, IL-10, IL-17 and IFNγ were present only at very low levels (Fig. 6.5B). Although at first viewing IL-4 and IL-10 levels induced following oral SEA administration appeared lower than the amounts produced by PBS-treated animals, this was not significant (Fig. 6.5B). IFNγ production was above background levels, but only at the highest SEA concentration or in mice receiving whole parasite eggs, this was also only a trend and non-significant in these groups (SEA 100μg: P=0.0870, Eggs: P=0.0832)(Fig. 6.5B). Analysis of the polyclonal response (stimulated by αCD3 treatment) indicated that, whilst administration of 50μg SEA enhanced the ability of MLN T cells to produce IL-5 and inhibited IFNγ, there was little evidence of a dose-response to SEA (Fig. 6.5C). Egg administration also inhibited polyclonal IFNγ production by MLN cells (Fig. 6.5C). Induction of polyclonal IL-4, IL-10, IL-13 and IL-17 were not significantly altered in any condition, when compared to levels from control (PBS-treated) mice (Fig. 6.5C).

As schistosome eggs are potent inducers of Th2 responses when administered by other routes (e.g. subcutaneously, i.p., i.v. to lungs (Chiaramonte *et al.*, 1999; McKenzie *et al.*, 1999; Phythian-Adams *et al.*, 2010; Sabin *et al.*, 1996)), the muted response following oral administration indicated this does not provide a good route to generate an egg-specific response. This is perhaps not surprising given that, during patent infection, eggs transit from the mesenteric vessels through the serosal side of the gut into the lumen. Thus, this

route of egg administration is very different from normal egg transit. For this reason, we decided to develop an alternative method of egg delivery, which involved surgical exposure of the small intestine under general anaesthesia, followed by injection of eggs into the subserosa of the intestinal wall. This route of egg administration is more akin to the natural route of egg transit, though of course not perfect, given that some mechanical disruption of the tissue does occur. This model was developed in collaboration with Simon Milling's lab at the University of Glasgow.

In contrast to oral administration (Fig. 6.5), subserosal egg injection induced a strong T cell response in the MLN 7d after injection (Fig. 6.6B). Although IL-4 production was relatively low, there was significant Ag-specific IL-5, IL-13 and IL-10 secretion by MLN cultures from egg-injected mice, compared to animals injected with a PBS control (Fig. 6.6B). Some level of IL-17 and IFNγ was also induced, but at variable levels. Induction of an egg-specific response was not restricted to the draining LN, as illustrated by Ag-restimulation of LP cells following egg injection. Significant levels of IL-4 and IL-5 were induced by egg injection, and LP cells also produced large quantities of IL-13 but the levels were more variable (Fig. 6.7B). IL-17 secretion was low-level but significant, whilst IL-10 and IFNγ production was variable between egg-injected animals (Fig. 6.7B).

In order to assess cytokine production specifically by LP $CD4^+$ T cells in response to subserosal egg injection, we carried out a pilot experiment assessing the expression of huCD2 by T cells from KN2 reporter mice was analysed by flow cytometry. These mice have an IL-4 allele replaced by human CD2, thus IL-4-producing cells can be identified by the expression of huCD2 on their surface (Mohrs *et al.*, 2005). Although not reaching significance with these low numbers of mice in this initial experiment, 2 out of 3 egg-injected animals displayed an increase in the proportion of $huCD2^+$ $CD4^+$ T cells in the LP (Fig. 6.7C). There was no significant difference in the percentage of $huCD2^+$ $CD4^+$ T cells present in the MLNs or PPs of PBS or egg-treated animals (Fig. 6.7D). To evaluate the production of other cytokines in the LP, in a separate pilot experiment ICC was performed following polyclonal stimulation with PMA/Ionomycin. Due to the low numbers of animals and the variability in cytokine production between mice in this initial experiment, there was no significant increase in IL-4, IL-5 and IL-13 production by LP $CD4^+$ T cells following egg injection (Fig. 6.8B-C). However, these Th2 cytokines were strongly induced in some mice

following egg injection, which was not seen in animals in the PBS-treated group. Fig. 6.8B depicts plots from animals where the subserosal egg injection was deemed successful, based on the percentage of TCRβ$^+$ CD4$^+$ cells expressing the Th2 cytokines, IL-4, IL-5 and IL-13. The proportion of intestinal CD4$^+$ T cells expressing IFNγ did not change (Fig. 6.8B-C), and there was no discernible alteration in the percentage of CD4$^+$ T cells in the MLN producing any of the cytokines that were assessed (Fig. 6.8C).

The above experiments, especially Ag-specific restimulations, show that subserosal schistosome egg injection clearly initiates a strong Th2 response in the small intestine. To begin to address the role of DCs in this response, we used CD11c-DOG mice to establish whether CD11c$^+$ cells are required. CD11c-DOG mice express a BAC transgene that includes the human diptheria toxin receptor under control of the CD11c promoter, administration of diptheria toxin (DTx) leads to the depletion of CD11c-expressing cells (Hochweller *et al.*, 2008). DTx can be administered to CD11c-DOG mice for up to 12 days with effective ablation of CD11c$^+$ cells (Hochweller *et al.*, 2008). CD11c$^+$ cells were depleted daily for 2 days prior to and 6 days following subserosal egg injection, and tissues were harvested on day 7. LP and MLN CD11c$^+$ DC populations were almost completely ablated in DTx-treated animals (Fig. 6.9B & D). Due to poor tissue preps in this experiment, the CD103$^-$ LP DC populations could not be detected in PBS-treated animals in this experiment (Fig. 6.9B). In line with a published study using CD11c-DTR mice (Varol *et al.*, 2007), the CD11c$^+$ LP MΦ population was also depleted in CD11c-DOG mice (Fig. 6.9B-C). Induction of egg-specific IL-4, IL-5, IL-10, IL-13 and IFNγ, but not IL-17, was dramatically ablated in the MLN of DTx-treated mice (Fig. 6.10), indicating that effective polarisation of this response is dependent on CD11c$^+$ cells. Together, this work demonstrates that subserosal egg injection robustly induces an egg-specific Th2 response in the LP and the MLN, and this is dependent on CD11c$^+$ cells.

6.2.3 The development of patent *S. mansoni* infection and the immune response are normal in the absence of MLN

The next steps in this project will be to determine the identity of the CD11c$^+$ cells that we have shown to be critical in the intestinal response to schistosome eggs (Fig. 6.10). As introduced above, we plan to help define which CD11c$^+$ cell type is key for induction of the egg specific Th2 response by cannulating the lymph draining the intestine, and

characterising the types of cell that migrate from these tissues, their activation status and function (Cerovic *et al.*, 2012). For this surgical technique, prior to cannulation, the mesenteric lymph nodes are excised, in order to shunt the intestinal flow into the thoracic duct, which can then be cannulated (Milling *et al.*, 2006). As a first important step towards future cannulation experiments, in order to assess whether mesenteric lymphadenectomy (MLNX) would have any debilitating effect on the development of the parasite or the immune response during *S. mansoni* infection, we infected mice with cercariae 28 days after surgical removal of the MLN. From d35 post-infection, the weight of infected animals was monitored because severe disease is associated with significant weight loss (MacDonald *et al.*, 2002b). In fact, both groups of infected mice actually gained weight over the course of infection, with the MLNX group even gaining significantly more weight than their intact counterparts (Fig. 6.11B). Mice were killed and parasitaemia assessed on d56 of infection. Both infected groups had comparable worm burdens (Fig. 6.11C), and there was no difference in egg counts in the liver or intestine (Fig. 6.11D), or in the number of eggs produced per worm pair (Fig. 6.11E). The development of hepatomegaly and splenomegaly was comparable in intact and MLNX infected mice (Fig. 6.11F). These findings suggest that *S. mansoni* parasites develop normally in mesenteric lympadenectomised animals. Finally, MLNX mice produced Th2 responses that were indistinguishable from intact animals, with comparable IL-4, IL-5, IL-10 and IL-13 production in the liver (Fig. 6.12B) and spleen (Fig. 6.12B), and with IL-13 actually significantly upregulated in the spleen in the MLNX group. No IL-17 or IFNγ was detected in either group of infected mice. These data demonstrate that the immune response is intact in MLNX mice infected with *S. mansoni*. There was no impact on the total number of cells present in the liver or spleen of naïve or infected MLNX mice (Fig. 6.13A and Fig. 6.14A). MLNX did have some impact on the numbers of lymphocytes present in these tissues, however, with a minor but significant increase in the number of $CD4^+$ T cells in the liver of infected MLNX mice (Fig. 6.13C), and in the spleen of naïve MLNX animals (Fig. 6.14C). However, B cell and eosinophil numbers were unaffected (Fig. 6.13C and Fig. 6.14C).

6.2.4 Summary

- There was no discernible change to small intestine LP myeloid cell populations on d42 of *S. mansoni* infection (Fig. 6.2 & Fig. 6.4A)
- However, by d56 of infection there was an influx of inflammatory monocytes (Fig. 6.3A-B) and a reduction in the $CD103^+$ DC populations (Fig. 6.3A&C)
- The LP-resident APC populations also down-regulated their expression of activation markers at this stage of infection (Fig. 6.4B-C)
- Oral administration of schistosome eggs or SEA does not induce a detectable Th2 response in the MLN (Fig. 6.5)
- Subserosal egg injection stimulates an adaptive immune response in the MLN (Fig. 6.6) and LP (Fig. 6.7), primarily characterised by production of Th2 cytokines
- The induction of this response is dependent on $CD11c^+$ cells (Fig. 6.10)
- *S. mansoni* parasites developed a patent infection in MLNX mice (Fig. 6.11), and a comparable level of immune activation to that of intact infected animals (Fig. 6.12)

6.3 Discussion

The small intestine is a major site of Ag challenge and inflammation during schistosome infection. Our long-term goal is to address the importance of specific DC subsets and IFN-I in induction, maintenance and regulation of immunity and inflammation in the intestines. The results presented in this chapter represent the beginning of this work, providing a solid foundation for future studies investigating the role of intestinal DCs in the induction of the immune response to *S. mansoni* eggs.

Successful purification of DCs from the intestine of infected mice is complicated, due both to high levels of mucus production and egg-induced intestinal pathology, which together results in excessive cell death. These techniques were not standard in our lab so we spent some time optimising the isolation of live cells from murine intestinal tissues during *S. mansoni* infection. We showed that during acute *S. mansoni* infection LP DC subsets did not display increased surface activation, and may in fact have a more muted activation level as infection progresses.

We then developed a more controlled approach to be able to dissect the immune response to schistosome eggs delivered synchronously to the intestine. Direct challenge with eggs injected into the serosa of the small intestine induced a strongly Th2 polarised cytokine response in both the MLN and the intestinal tissue itself, and the induction of this response was dependent on the presence $CD11c^+$ cells. This novel model of egg deposition in the intestine will enable future studies to directly assess the phenotype and function of the different DC subsets during induction of the egg-specific response.

Analysis of *S. mansoni* infection in mesenteric lymphadenectomised mice demonstrated that parasite development and immune activation occurs normally in the absence of the MLN. This important preliminary finding will now allow characterisation and functional studies of the cell subsets that are mobilised from the intestine during infection, through cannulation studies that will also be used in the subserosal egg injection model.

6.3.1 Downmodulation of myeloid cell activation with progressive *S. mansoni* infection

The myeloid populations identified in the steady state LP appeared identical to the subsets previously described in the literature (Fig. 6.1)(Bogunovic *et al.*, 2009; Varol *et al.*, 2010). As well as identifying the more abundant $CD103^+$ $CD11b^+$ and $CD103^+$ $CD11b^-$ DC subsets, we also identified $CD103^-$ $CD11b^-$ LP DCs, a population that is often overlooked, perhaps because of its low-level expression of CX3CR1 (Cerovic *et al.*, 2012). These cells could be mistaken for MΦs. However, it has been shown that they are present in intestinal lymph (Cerovic *et al.*, 2012). Key to the APC function of DCs is their ability to migrate efficiently to the draining LN following exposure to Ag, in order to present Ag to naïve T cells resident in the LN, and to polarise adaptive immune responses, MΦs do not display the same migratory capacity (Geissmann *et al.*, 2010a; Randolph *et al.*, 2008), and are not present in intestinal lymph (Cerovic *et al.*, 2012). $CD103^-$ $CD11b^{+/-}$ DCs isolated from the small intestine of naïve mice can prime T cells effectively and expand in response to Flt3-L (Cerovic *et al.*, 2012). However, it still needs to be shown definitively whether the $CD11b^-$ DCs alone can prime. Although the highest level of expression of CX3CR1 is found on LP MΦ, DC subsets in the LP also express this marker to some degree (Fig. 6.1C) (Cerovic *et al.*, 2012). Given that CX3CR1 can only be reliably identified using GFP-reporter mice, it is clear that it does not provide an ideal tool for delineation of the myeloid populations in the LP of e.g. gene deficient mice. Recent studies have identified CD64 (FcγR1) as a more discriminating marker of intestinal MΦ (Bain *et al.*, 2012; Tamoutounour *et al.*, 2012), which should be used in future experiments.

$CX3CR1^{hi}$ cells in the small intestine LP were initially described to be a DC population (Niess *et al.*, 2005). However, more recent studies have revealed these cells to be MΦ, a non-migratory population derived from monocytes in response to M-CSF (Bogunovic *et al.*, 2009; Schulz *et al.*, 2009). Functional studies performed by Schulz *et al*. demonstrated that, whilst $CX3CR1^+$ LP MΦs readily take up Ag *in vivo*, they are unable to prime Ag-specific $CD4^+$ T cells at all, and stimulate only low-level proliferation of $CD8^+$ T cells. Nevertheless, this population may be important in Ag sampling from the intestinal lumen (Niess *et al.*, 2005; Schulz *et al.*, 2009), which may help to maintain immunoregulation in the tissues in the steady state (Bain *et al.*, 2012; Murai *et al.*, 2009), and aid in bacterial clearance following infection with pathogenic species such as *S. typhimurium* (Niess *et al.*,

2005). However, priming of *S. typhimurium*-specific T cell responses still relies on DCs, not MΦs (Bogunovic *et al.*, 2009). High expression levels of MHC II (Fig. 6.1B), as well as co-stimulatory molecules, often with a higher gMFI than the DC subsets (Fig. 6.1E-F & Fig. 6.3), illustrate the potential for LP MΦ to interact with T cells in the intestinal tissue. Thus, the MΦ population should not be ignored when considering the role of myeloid populations in the LP.

There was no measurable alteration in the myeloid cell populations present in the LP on d42 of *S.mansoni* infection (Fig. 6.2D-F), indicating that initial egg deposition does not cause a significant efflux of DCs. This finding is in agreement with the hypothesis presented in chapter 3, that SEA-stimulated DCs traffic to the dLN at a similar rate to steady state DC migration. At this timepoint of infection, there was also no discernible increase in the expression level of co-stimulatory molecules on LP DCs or MΦs (Fig. 6.4A). Future cannulation studies will allows us to study the DCs leaving the intestinal tissue following egg challenge. As this population should include Ag-experienced cells, it will be interesting to assess their surface phenotype in comparison to the LP DC populations as a whole. Assessment of DCs present in the MLN during *S. mansoni* infection will also provide information on the phenotype of Ag-experienced cells, as a large proportion of the DCs in the MLN will have migrated from the intestinal tissue (Jakubzick *et al.*, 2008; Satpathy *et al.*, 2013). However, this data must be used with caution, as it is difficult to identify which cells have migrated from those that are resident in the MLN.

From our work with FL-cDCs (Chapter 3), we would perhaps have expected an upregulation of co-stimulatory molecules on LP DC subsets during *S. mansoni* infection. However, by comparison of gMFI data for the expression of CD40 and CD80 by unstimulated $CD24^+$ FL-cDCs (Fig. 3.7B) and $CD103^+$ $CD11b^-$ LP DCs from a naïve mouse (Fig. 6.4), it is clear that the basal expression level of these markers is 3-6 fold higher on the LP-resident subset than on FLDCs. Although this is not a direct comparison as these experiments were carried out independently, this may explain the failure of egg deposition in the intestine to induce DC activation, as the basal activation level of DCs *in vivo* in this tissue site appears higher than the surface phenotype stimulated in FLDCs by SEA *in vitro*. Another possibility is that the intestines are, by necessity, a more highly regulated

tissue than the spleen, with constitutively high levels of TGFβ, IL-10 and Tregs. This may keep in check any activation in response to eggs and egg Ags.

By d56 of infection, there were marked changes in the myeloid populations in the LP that were not seen at the earlier timepoint, and this may reflect the dramatic increase in intestinal egg burden at this stage (Turner *et al.*, 2012), as well as increased inflammation and tissue damage (Pearce and MacDonald, 2002). An influx of $CD11b^{+}$ $Ly6C^{hi}$ inflammatory monocytes was observed in the LP by d56 of *S. mansoni* infection (Fig. 6.3A-B). Similar inflammatory monocyte influxes have been described in both colitis models and during infection with parasitic protozoa in the small intestine (Grainger *et al.*, 2013; Platt *et al.*, 2010; Zigmond *et al.*, 2012). There was a stark reduction in $CD103^{+}$ $CD11b^{+}$ DCs in the LP by d56 of infection (Fig. 6.3C). This unexplained phenomenon has been described previously in the inflamed LP (Grainger *et al.*, 2013). $CD103^{+}$ $CD11b^{+}$ DCs are thought to be the primary LP population that migrates to the MLN and primes $CD4^{+}$ T cells (Bogunovic *et al.*, 2009; Schulz *et al.*, 2009). As such, they represent the majority of DCs present in intestinal lymph (Cerovic *et al.*, 2012). It is therefore possible that this population is mobilised from the LP by d56 of schistosome infection. This could be tested simply by comparing the proportions and numbers of this DC subset present in the MLN, i.e. is there an increase in $CD103^{+}$ $CD11b^{+}$ DCs by d56 of infection? Cannulation studies at this stage of infection would also indicate whether there was an increase in mobilisation of this subset. The reduction in $CD103^{+}$ $CD11b^{+}$ DCs may also reflect a disruption in the turnover of LP DCs during infection, as $CD103^{+}$ DCs are replaced by precursors recruited from the blood more frequently than $CD103^{-}$ subsets (Bogunovic *et al.*, 2009).

A reduction in numbers of $CD103^{+}$ DCs in the SI LP may impact on the development of T cell responses at this site, particularly Treg differentiation, as the $CD103^{+}$ DC subset preferentially induces Treg differentiation in the steady state (Coombes *et al.*, 2007), although it is not yet known if this is also the case during helminth infection. Work from Yasmine Belkaid's lab also demonstrates a reduction in Treg numbers in the small intestine during protozoal infection (Oldenhove *et al.*, 2009), along with loss of $CD103^{+}$ DCs (Grainger *et al.*, 2013). This suggests that Treg numbers may be reduced in the small intestine during *S. mansoni* infection, although this hypothesis needs testing directly. A study of the granulomatous response in the colon identified an increase in Treg numbers

during chronic infection (Turner *et al.*, 2011). However, nTreg numbers are not altered in the MLN during *S. mansoni* infection (Baumgart *et al.*, 2006). Weinstock *et al.* have previously demonstrated that granulomas in the ileum are much smaller than other intestinal granulomas, a finding which may suggest a tissue-site specific impairment of the normally 'florid' acute granuloma response, this may relate to the highly regulatory phenotype of the ileum (Powrie, 2004), and would contrast with the hypothesis that Treg responses are deficient at this site during the acute stage of *S. mansoni* infection. Thus, a comprehensive study of the numbers of Tregs in the small intestine over the course of *S. mansoni* infection is required, as well as functional characterisation of these cells in this setting. A study of Tregs on d63 of *S. mansoni* infection has shown that $Foxp3^+$ cells are localised around the periphery of granulomas in the intestine, although the authors do not state what site(s) in the intestine was included in this analysis (Layland *et al.*, 2010).

An influx of $CD11b^+$ $Ly6C^{hi}$ monocytes, as seen by d56 of *S. mansoni* infection, is primarily associated with an exacerbation of intestinal inflammation (Platt *et al.*, 2010; Zigmond *et al.*, 2012). However, a recent study indicates that this cell population can also regulate innate immune activation, limiting neutrophil-mediated pathology in the intestine in a prostaglandin E_2 (PGE_2) -dependent manner during *Toxoplasma gondii* infection (Grainger *et al.*, 2013). It is not yet known whether infiltrating monocytes promote or limit inflammation and immune responses in the helminth-infected intestine, or whether they can influence DC phenotype and function. Intriguingly however, PGE_2 has also been shown to inhibit retinoic acid production by DCs (Stock *et al.*, 2011), which is required for Treg differentiation in the steady state intestine (Coombes *et al.*, 2007). This may explain the reduction in Treg numbers during protozoan infection in the small intestine (Oldenhove *et al.*, 2009). In support of the hypothesis that $CD103^+$ DC function is impaired during parasite infection, preliminary data from our lab indicates that activity of RALDH, the enzyme responsible for retinoic acid (RA) generation, is down-regulated in the $CD103^+$ DCs that remain in the SI LP on d49 of *S. mansoni* infection in mice infected with 180 cercariae (Lucy Jones and LMW). It should be noted that it has not yet been shown that $CD103^+$ DCs function to promote Treg differentiation in helminth infection, or whether RA, and RA-producing cells, play an entirely different role in these settings. It is likely that the function of $CD103^+$ DCs may change following exposure to helminth Ags, particularly in

light of recent studies that have shown these cells are involved in Th17 polarisation against pathogenic bacteria (Persson *et al.*, 2013; Satpathy *et al.*, 2013).

It cannot be ruled out at this stage that changes in the numbers and phenotype of myeloid cell populations described on d56 of *S. mansoni* infection may be caused by cell damage or death, given that there is severe immunopathology at this stage of infection, which may render cells more susceptible to death during tissue preparation and cell isolation. For this reason, it would be informative to assess the proportions of myeloid cells undergoing apoptosis from the intestine of naïve mice, and from the tissues of animals at different stages of *S. mansoni* infection. Further, these characterisation experiments need to be repeated with larger sample sizes to enable statistical analysis of the observed changes.

Studies of inflammatory monocytes have primarily focused on the effect they have on Th1/ Th17 immune responses within the intestine. However, recent studies have also highlighted that in some settings these cells can downregulate Ly6C expression, and subsequently migrate in the intestinal lymph towards the MLNs. These cells can also induce Ag-specific T cell proliferation *in vitro* (Zigmond *et al.*, 2012). For this reason, a potential role of these cells in T cell polarisation during *S. mansoni* infection should not be overlooked. Again, this can begin to be addressed by characterising the cell populations migrating from the intestine to first determine if they are present in the intestinal lymph during infection.

The importance of intestinal epithelial cells (IECs) in orchestrating the immune response against gastrointestinal helminths has been demonstrated (Artis and Grencis, 2008; Cliffe *et al.*, 2005), and this includes production of tissue factors such as TSLP, IL-25 and IL-33 which promote Th2 induction and worm expulsion (Humphreys *et al.*, 2008; Rimoldi *et al.*, 2005; Saenz *et al.*, 2010; Zaph *et al.*, 2007). It is therefore possible that increasing egg-induced damage around d56 of *S. mansoni* infection elicits cytokine secretion from IECs that influence DC activation. TSLP directly induces human DCs to produce IL-10 and enhances costimulatory molecule expression, (Rimoldi *et al.*, 2005), resulting in increased Th2 responses (Rimoldi *et al.*, 2005; Soumelis *et al.*, 2002). As such, more likely candidates for down-regulating DC activation in the intestine during *S. mansoni* infection are TGFβ and IL-10. TGFβ is produced by both IECs (Zeuthen *et al.*, 2008), and DCs

themselves, particularly by the CD103^{+} DC populations (Coombes *et al.*, 2007). IEC-derived TGFβ, in conjunction with IEC-TSLP, have been shown to induce a tolerogenic phenotype from DCs, at least *in vitro* (Zeuthen *et al.*, 2008).

Blockade of both TGFβ and IL-10 indicate that production of these regulatory cytokines plays an important role in protecting against severe liver damage during *S. mansoni* infection (Herbert *et al.*, 2008). IL-10 production from T effectors and innate cells, as well as Tregs, is integral to the regulation of aberrant immune activation in the liver of *S. mansoni* infected mice (Dewals *et al.*, 2010; Hesse *et al.*, 2004). However, inhibition of IL-10 and TGFβ did not in fact lead to exacerbation of intestinal immunopathology (Herbert *et al.*, 2008). The authors suggested that control of aberrant immune activation in the intestine is controlled by alternatively-activated MΦs (Herbert *et al.*, 2004), and went on to demonstrate the importance of Arginase-I production by AA MΦs in suppressing inflammation in the gut during acute *S. mansoni* infection (Herbert *et al.*, 2010; Pesce *et al.*, 2009). Arginase-I functioned to limit production of pro-Th1/ Th17 cytokines such as IL-12/IL-23p40 and IL-6 (Herbert *et al.*, 2010), and also served to inhibit T cell turnover (Pesce *et al.*, 2009). Thus, it is possible to imagine that Arginase-I, which is also produced by DCs (Chang *et al.*, 2013) could additionally limit DC activation and promote immune regulation. Whilst Herbert *et al*. (2010) investigated the importance of Arginase-I during *S. mansoni* infection using Arginase-I deficient BM chimeras, this does not address the importance of DC-derived Arginase-I and whether this may play a role in regulating immune activation in the intestine during infection.

Whether or not the Ly6C^{hi} inflammatory monocytes that are recruited to the intestine during *S. mansoni* infection become alternatively-activated remains to be determined. It is also possible that intestinal AAMΦs induced during *S. mansoni* infection are derived from proliferation of the CX3CR1^{+} LP-resident MΦ populations. In this regard, in infection with the filarial nematode, *Litomosoides sigmodontis*, infiltrating monocytes do not contribute to the generation of AAMΦ in the infection site until relatively late in infection, if at all (Jenkins *et al.*, 2011). Nevertheless, in schistosome infection we did not detect an influx of Ly6C^{hi} inflammatory monocytes until d56 of *S. mansoni* infection, and studies indicate that AAMΦ are essential for protection at earlier timepoints than this (Herbert *et al.*, 2004; Herbert *et al.*, 2010), implying that CX3CR1^{+} LP MΦs are likely to be the primary cell population

contributing to the pool of AAMΦs in the intestine in acute infection. If this were the case, this would highlight an important protective role for CX3CR1$^+$ LP MΦs in Th2 inflammation in helminth infection. One way to address this would be to investigate the presence of CX3CR1$^+$ cells in the intestinal granulomas of CX3CR1-GFP mice by microscopy. Given that these cells are also ablated by DTx treatment of CD11c-DTR mice (Varol *et al.*, 2007), it may also be possible to assess the importance of these cells in granuloma formation during *S. mansoni* infection in DTx-treated CD11c-DOG mice. The obvious downside to this method is that DCs would also be depleted.

6.3.2 DC-dependent Th2 induction in the small intestine following subserosal egg injection

As an initial approach to try to develop a more controlled model of schistosome egg Ag exposure in the intestines, we tried oral administration of SEA or schistosome eggs. However, preliminary experiments using a range of SEA doses or 2500 eggs, failed to elicit a Th2 response in the MLN 7d after oral gavage (Fig. 6.5B). Since we did not perform a full time course analysis, it remains possible that we missed the peak of Th2 response. However, the lack of any detectable IL-13 or IL-5, coupled with an actual decrease in IL-4 and IL-10, suggests this is unlikely to be the case (Fig. 6.5B). This is consistent with oral administration leading to the generation of tolerance against SEA/ eggs, rather than priming immune activation (Pabst and Mowat, 2012). In retrospect, this seems like an obvious outcome of oral administration of *S. mansoni* eggs/ SEA. However, there is some indication that this route of egg/ SEA delivery can enhance polyclonal Th2 (Fig. 6.5C) – perhaps a reflection of the Th2 'adjuvant' ability of SEA.

Subserosal egg injection proved to be a much more successful route of Ag challenge in the intestine, generating an egg-specific response in the draining LN (Fig. 6.6B) and in the LP tissue itself (Fig. 6.7B). Although there was only low-level IL-17 and IFNγ production stimulated by egg injection, particularly in the MLN, the IL-17 response in the LP was significantly above background (Fig. 6.7B). This reflects the fact that both Th17 and IL-17-producing innate cells are constitutively present in the LP in the small intestine (Cua and Tato, 2010; Ivanov *et al.*, 2006; Takatori *et al.*, 2009).

In contrast to the data from the SEA restimulation of MLN cells (Fig. 6.6), there was no detectable Th2 cytokine production above background from MLN $CD4^+$ T cells by ICC (Fig. 6.8C) or using the KN2 reporter mice (Fig. 6.7D). This may be due to the use of PMA/ Ionomycin to stimulate cytokine production, which is a very potent stimulator of all lymphocytes and may cause the death of Ag-specific T cells that are already activated, due to overstimulation. A much more relevant ICC protocol would use SEA to restimulate Ag-specific cells, however, production of cytokines following *in vitro* SEA restimulation is of course much lower than with a polyclonal stimulus, and there may not be enough Ag-specific T cells present to provide a reliable readout. An alternative method is to inject brefeldin prior to killing experimental mice, any T cells that are producing cytokines in the tissues will maintain their cytokine intracellularly, which may be detectable after isolation. However, this may also impact on the survival of cells during processing. Our preliminary KN2 data suggests that this reporter may be useful for identifying IL-4 production in the LP; however, there was no hint of $huCD2^+$ cells in the MLN. More extensive use of IL-4/ 10/ 13-GFP reporters, in conjunction with the KN2 mice, would allow us to assess whether there are cells present in the MLN and LP that are capable of making Th2 cytokines, without using any kind of stimulus, which can have artifactual effects on the cytokine readout.

We have some preliminary ICC and KN2 data that suggests that $CD8^+$ T cells and B cells do not make Th2 cytokines following subserosal egg injection, however, reporter mice will enable us to do a more comprehensive analysis of this. There are also a number of other cell populations whose contribution to cytokine production in response to subserosal egg injection or during *S. mansoni* we have not yet considered. These include ILC2s, which have been shown to be active in the intestine during helminth infection, acting as a source of Th2 cytokines, particularly IL-13 (Fallon *et al.*, 2006; Neill *et al.*, 2010; Saenz *et al.*, 2010). Other cell types to investigate for a role in cytokine production in the intestine are γδ T cells, NK cells and intraepithelial lymphocytes (IELs). A large proportion of IELs are γδ T cells, these cells are primarily associated with IFNγ production, but have been shown to produce IL-4 under specific culture conditions *in vitro* (Caccamo *et al.*, 2013). Further, these cells are expanded during infection with the intestinal helminth *Trichinella spiralis* (Bozic *et al.*, 1998), although their production of cytokines during helminth infection has not been shown. Similarly to γδ T cells, NK cells produce IFNγ during viral and bacterial

infection, but have also been shown to produce IL-13 during infection with *T. spiralis* (McDermott *et al.*, 2005), however, this population was characterised in SCID mice so it is difficult to know what contribution these cells make in immunocompetent mice.

Whilst surgery and subsequent microinjection into the intestinal tissue and manipulation of the organ may cause some level of immune activation, PBS injected control mice lacked any detectable response. As microinjection is a technically demanding procedure, it is perhaps not surprising to find variation in cytokine responses between mice (Fig. 6.6B & Fig. 6.7B). This may also account for the variability in the data from our attempts to characterise the T cell response in the LP. The LP is a very fragile tissue to work with and dead/dying cells can affect the readout. Thus, there is a suggestion that huCD2 expression on $CD4^+$ T cells is increased in the LP (Fig. 6.7C), but this did not reach significance (Fig. 6.7C). Similarly, the *ex vivo* restimulation ICC readout was also very variable (Fig. 6.8C). In a proportion of animals that received S. mansoni eggs subserosally, elevated levels of Th2 cytokines were detectable (Fig6.8B-C). It is hypothesised that these mice may have been more successfully dosed with eggs than those animals where no change in Th2 cytokine levels were detectable. To test this, it would be beneficial to perform egg count from the intestine to compare the antigen load between different mice. Thus our data strongly suggests that subserosal egg injection is a good technique to induce Th2 cytokine production from $CD4^+$ T cells in the intestinal tissue, but additional experiments are required to confirm this.

Previous work from our lab has shown that the induction of the Th2 response in the liver and spleen during *S. mansoni* infection is dependent on $CD11c^+$ cells (Phythian-Adams *et al.*, 2010). Similarly, MLN Th2 responses following infection with the gastrointestinal nematodes *H. polygyrus* and *Nippostrongylus brasiliensis*, also requires $CD11c^+$ cells (Smith *et al.*, 2012; Smith *et al.*, 2011).

In our experiments we use CD11c-DOG mice generated by Hochweller *et al*. (2008) rather than the CD11c-DTR mouse model developed by Steffen Jung (Jung *et al.*, 2002). Both of these mouse models express a DTR transgene under the control of the CD11c promoter, however, they differ somewhat in the transgenic construct used, also, DOG mice express the human DTR, whilst CD11c-DTR mice express a simian receptor. The CD11c-DTR

mice only tolerate 1-3 administrations of DTx before they succumb to toxicity, most likely due to expression of the DTR on other cell populations, such as IECs (van Blijswijk *et al.*, 2013; Zammit *et al.*, 2005). To avoid this BM chimeras are generated, WT irradiated animals reconstituted with CD11c-DTR BM can survive longer exposure to DTx (Zaft *et al.*, 2005; Zammit *et al.*, 2005). The use of a BAC construct by Hochweller *et al*. means that the expression of the transgene is more restricted in CD11c-DOG animals and mice tolerate up to 11 days of DTx administration without ill effect. After this timepoint it is thought the development of DTx-specific antibodies render DTx treatment ineffectual, and from this time onwards levels of depletion are reduced (Hochweller *et al.*, 2008). Both of these models deplete a number of other cell populations, including some monocyte and MΦ populations, plasmablasts, activated T cells and NK cells (van Blijswijk *et al.*, 2013). This is particularly problematic in the intestine, where the CD11c-expressing LP MΦ population is ablated.

Our results with subserosal injection of *S. mansoni* eggs into CD11c-DOG mice show that $CD11c^+$ cells are also required for the egg-specific response in the MLN, as DTx treatment appeared to ablate Th2 differentiation (Fig. 6.10). Repeat experiments will confirm this statistically, and also reveal whether LP responses are reduced in the absence of $CD11c^+$ cells. CD11c depletion in CD11c-DOG mice removed both LP DCs and MΦ, so it is unclear from these experiments which myeloid population is required for the induction of the egg-specific response in the LP and MLN. This could be addressed using a more specific depletion model such as zDC-DTR mice, where DTx administration selectively depletes populations that express the cDC-specific TF Zbtb46 (Meredith *et al.*, 2012). Additionally, depletion type experiments also do not tell us whether DCs migrating from the LP actually prime the MLN response, or what contribution Ag draining directly to the LN, and MLN-resident cells, make to presentation to and polarisation of Th2 cells. This is why lymphatic cannulation will be such a useful tool, enabling us to identify whether egg deposition induces DC migration from the LP, and to characterise these cells both phenotypically and functionally. So far, we have found it difficult to identify whether DCs exposed to schistosome egg Ags migrate more readily than cells in the steady state (Chapter 2). Cannulation after subserosal egg injection will provide an ideal way to address this question, as we will be able to identify whether egg injection increases the number of cells migrating away from the intestine, compared to controls.

If infection or subserosal egg injection does stimulate increased DC migration, this will provide important information on the contribution of the different DC subsets in orchestrating the egg-specific Th response. Previous studies have identified the $CD103^+$ $CD11b^+$ DC subset as the primary migratory population that primes T cell responses in the MLN during the induction of Th1 and Th17 responses (Jaensson *et al.*, 2008; Persson *et al.*, 2013; Satpathy *et al.*, 2013; Schulz *et al.*, 2009). However, $CD103^+$ $CD11b^-$ cells (30%) and $CD103^-$ $CD11b^{+/-}$ cells (15%) make up substantial proportions of DCs in intestinal lymph (Cerovic *et al.*, 2012), thus a potential role for these subsets cannot be discounted. We plan to assess the importance of $CD103^+$ $CD11b^-$ DCs using *Batf3*$^{-/-}$ mice, which are deficient in $CD8\alpha^+$ DCs (Hildner *et al.*, 2008), and selectively lack the $CD103^+$ $CD11b^-$ DC subset in the LP and other nonlymphoid tissues in the steady state (Edelson *et al.*, 2010). However, compensatory mechanisms can partially rescue $CD8\alpha^+$ DC differentiation in the spleen and $CD103^+$ $CD11b^-$ DCs in the tissues in *Batf3*$^{-/-}$ mice during inflammatory responses (Tussiwand *et al.*, 2012). This response seems to be particularly driven by Th1 cytokines, such as IL-12 (Tussiwand *et al.*, 2012), thus it is entirely possible that there will be no compensation in the intestines of *Batf3*$^{-/-}$ mice during *S. mansoni* infection. An alternative approach would be the use of *Irf8*$^{-/-}$ mice, which also lack both $CD103^+$ $CD11b^-$ and $CD8\alpha^+$ DCs (Edelson *et al.*, 2010; Ginhoux *et al.*, 2009; Tamura *et al.*, 2005). IRF4 is required for $CD11b^+$ DC differentiation (Suzuki *et al.*, 2004; Tamura *et al.*, 2005), in the absence of this TF in CD11c cells, the $CD103^+$ $CD11b^+$ DC subset are ablated in the LP and MLN (Persson *et al.*, 2013). The caveat of these mice is that the $CD103^-$ $CD11b^+$ population is also reduced in the steady state LP and in the MLN, however this population is restored under inflammatory conditions (Persson *et al.*, 2013). Development of this subset is also dependent on Notch2 signalling and is completely ablated in *Cd11c-cre.Notch2*$^{fl/fl}$ mice (Satpathy *et al.*, 2013). These mouse models may be useful to assess the role of this particular DC subset in the induction of the egg-specific response following subserosal egg injection or during patent *S. mansoni* infection.

The surgical egg injection model offers several advantages over natural infection. From our analysis of LP DCs and MΦ during *S. mansoni* infection (Fig.6.2- Fig.6.4), we would perhaps not expect to see any change in the surface phenotype of these cells following subserosal egg injection. However, this model provides a unique opportunity to study how

intestinal DCs respond to egg deposition, as it is impossible to know precisely when eggs have been laid during patent infection. DC activation should be assessed at early timepoints after injection. Our timecourse experiments using FLDCs suggest that surface activation in response to SEA is detectable on these cells from 6h onwards (Fig. 3.4A). We have also detected gene expression changes in splenic DCs within 12h after intravenous SEA injection (Fig. 5.1D-E). These data suggest that, if egg injection were to stimulate DC activation, phenotypic changes may be detectable within the first few hours.

There are a huge number of exciting potential experiments that would be an extension of the preliminary data we have from the subserosal egg injection model. The priority is certainly to identify which $CD11c^+$ cell populations are involved in the egg-specific Th2 response in the intestine and MLN, using the depletable or knockout models discussed above. We also want to do these same experiments during patent infection. Prior to performing cannulation experiments during patent infection, it will be informative to perform these studies in the subserosal egg injection model to assess the DC populations mobilised from the intestine following injection. Prior to commencing these studies we need to do a similar experiment as performed in MLNX *S. mansoni* infected mice, in order to assess whether removal of the MLN impacts on the development of the egg-specific response in this setting.

We have begun to address the cytokine production in the LP and MLN following egg injection, in both settings we plan to use cytokine reporter mice to fully investigate which cell populations are actively involved in the cytokine response. Whilst initial experiments have concentrated on subserosal LP egg injection, *S. mansoni* worms primarily lay their eggs in the vasculature that surround the PP (Turner *et al.*, 2012). Thus, egg injection into the PP could be incorporated into this model to increase the relevance to patent infection. Currently, we have been using PBS as a control in the egg injection experiments, a better control would be latex beads, which may better mimic the presence of a solid mass disrupting the tissue lining of the gut.

The subserosal egg injection model could also be developed further to allow controlled/ refined assessment of intestinal granuloma formation. Currently, the primary model of granuloma formation around schistosome eggs is a pulmonary model, which involves

intravenous injection of eggs (Edungbola and Schiller, 1979; Eltoum *et al.*, 1995). A similar method modelling *S. haematobium* egg deposition in the bladder wall has been developed (Fu *et al.*, 2012). Injecting live *S. haematobium* eggs into the bladder induced pathology and an influx of innate effector cells, such as eosinophils and neutrophils (Fu *et al.*, 2012). By d28 organised granulomas were detectable in the bladder wall. Granuloma formation can be induced more quickly by prior sensitisation with eggs or SEA intraperitoneally, which is the favoured method for inducing granuloma formation in the lung in the pulmonary model (Joshi *et al.*, 2008; Wynn *et al.*, 1997). Pre-sensitisation prior to subserosal egg injection may actually provide a better mimic of active infection, as in this setting the host is exposed to systemic parasite Ag from the larval, worm and egg stages that will influence the response to egg deposition, due to prior APC and T cell activation by these schistosome Ags. Thus, pre-sensitisation followed by subserosal egg injection would provide a novel model with which to characterise the development of granulomas against *S. mansoni* eggs in a highly relevant tissue site. With the addition of the different DC-depleting mouse models we will also be able to characterise the role of the DC subsets in the formation of granulomas in the intestine.

Fu *et al.* (2012) used freshly isolated live eggs in their experiments, whilst we have only injected dead eggs up to this point. The distinct advantage of injecting live eggs is that it is much more analogous to patent infection, as live eggs actively secrete Ags that stimulate the immune response, including omega-1, the primary Th2-polarising Ag in SEA (Everts *et al.*, 2009; Steinfelder *et al.*, 2009). For this reason, live eggs are more likely to stimulate eosinophilia and the formation of organised granulomas. A disadvantage of injecting live eggs is that they must be harvested and purified from infected animals directly prior to injection, which must be worked in to an already complex experimental setup. Another possibility is harvest of freshly laid eggs from cultured perfused adult worms, this is also technically challenging, however, as worms do not live or continue to lay eggs very long *in vitro* (Galanti *et al.*, 2012). However, a better understanding of the nutritional requirements of cultured female worms for egg laying, in particular, the importance of fatty acid metabolism (Huang *et al.*, 2012), has the potential to improve fecundity of schistosome parasites *in vitro*, which could be used as a source of live eggs for injection. The benefit of using eggs from cultured worms is that it would require no tissue processing on the day of injection. A lack of any tissue processing (above and beyond worm perfusion) is also

beneficial, as eggs will not be negatively impacted by mechanical or enzymatic disruption, which is required for the purification of eggs from tissues. It has previously been shown that extraction methods can alter the immunogenic properties of schistosome eggs (Hirata *et al.*, 1991).

Overall, we have developed a model of synchronous *S. mansoni* egg delivery to the intestine that polarises an Ag-specific Th2 response in the LP and MLN. Induction of the Th2 response in this model depends on $CD11c^+$ cells, thus providing an ideal system to go on to dissect the roles of the different myeloid cell populations in priming immune activation and Th2 polarisation against egg deposition. Future experiments with this model will inform and enhance our studies of the intestinal response to patent *S. mansoni* infection.

6.3.3 *S. mansoni* infection and the consequent Th2 response develop normally in mice lacking MLN

Future work will include more detailed analysis of the phenotype and function of intestinal DCs during helminth infection; particularly, we plan to use the intestinal site to test the relevance of our findings with FLDCs and IFN-I. Although our egg injection models provide great models with which to investigate these areas, it is also necessary to examine whether these findings are reproducible in the more complex setting of infection. In order in the future to be able to develop lymphatic cannulation to study the DCs mobilised from the intestine during *S. mansoni* infection, we first had to address whether mesenteric lymphadenectomy would negatively impact on parasite development or the induction of the immune response. Thus, we infected intact or lymphadenectomised mice with *S. mansoni* cercariae 28d after surgery, and assessed parasitology and immunology in these animals on d56 of infection.

The development of adult worms was clearly unaffected by the absence of MLN, as the number of worm pairs was comparable in intact and MLNX mice (Fig. 6.11C). The adult worms reside and deposit their eggs within the mesenteric blood vessels (Gryseels *et al.*, 2006), thus it was possible that surgical removal of the MLN could cause damage to the vasculature and negatively impact on worm fecundity or egg deposition. However, this proved not to be the case as egg burden (Fig. 6.11D) and egg production per worm pair

(Fig. 6.11E) was comparable in intact and MLNX infected mice. Similarly, there was no defect in the Th2 response in the liver or spleen of MLNX animals (Fig. 6.12). The only significant difference between the infected groups was an increase in Ag-specific IL-13 induction in the spleen of MLNX mice (Fig. 6.12B). Following mesenteric lymphadenectomy, the lymphatic vessels heal, allowing intestinal lymph to flow into the thoracic duct (Milling *et al.*, 2006). The thoracic duct drains into the blood system, thus any cells migrating from the intestine in MLNX mice will eventually reach the spleen. This may explain why IL-13 levels are enhanced in the spleen of infected MLNX animals, although there was no concurrent increase in the other Th2 cytokines in these mice (Fig. 6.12B).

Given that parasite development is not affected in the absence of the MLN, it is perhaps unsurprising that the immune response is also intact. The immune system is clearly able to compensate for the absence of this secondary lymphoid organ. Studies of *S. mansoni* infection in splenectomised mice indicate that the immune response is also intact in mice lacking a spleen (Arruda *et al.*, 1993; Hood and Boros, 1980; Mahmoud and Woodruff, 1978). As outlined above, it is likely that the spleen is the primary organ to compensate for the lack of MLN following lymphadenectomy. We have yet to establish whether the Th2 response or pathology is altered in the intestine itself in MLNX mice. DCs in the MLN uniquely prime gut-homing T cells (Johansson-Lindbom *et al.*, 2005), so it is possible that there may be some defect in the generation of these cells in MLNX mice. However, it is likely that LP DCs which are responsible for priming gut-homing T cells migrate to the spleen and are able to mobilise gut-tropic $CD4^+$ T cells from there instead. This could be assessed by analysing the expression of the gut-homing markers CCR9 and α4β7 on the T cells found in the spleen of MLNX mice during infection, particularly those that are producing Th2 cytokines. No weight loss was recorded for MLNX mice (Fig. 6.11B), indicating that the immune response was functional in both the liver (Fig. 6.12B) and the intestine of these mice, as cachexia is associated with severe hepatic and intestinal damage in *S. mansoni* infection (Brunet *et al.*, 1997; Herbert *et al.*, 2010). There was also no dramatic alteration in the immune cell populations in the liver or spleen during infection (Fig. 6.13 and Fig. 6.14), however, there was a minor increase in $CD4^+$ T cells in the livers of infected mice, and in the spleen of naïve MLNX animals. There was also an increase in $CD4^+$ T cells in the spleens of MLNX infected animals, although not significant, this

increase may account for the fact that IL-13 production was enhanced at this site (Fig. 6.12).

Having confirmed that acute *S. mansoni* infection develops normally, we can now go on to directly address whether myeloid cells are mobilised from the intestine during infection, and to characterise these cells. This will provide the first opportunity to elucidate the contribution of LP-resident DC subsets in the induction of Th2 inflammation in the intestine. These studies will be further enhanced by direct comparisons of the phenotype and function of the different DC subsets in the LP and lymph with those found in the MLN.
Experiments in this final chapter have demonstrated that $CD11c^+$ cells are likely to be essential to the induction of Th2 responses in the intestine. However, DCs and MΦs in the SI LP downregulate surface expression of molecules required for T cell polarisation during the acute stage of *S. mansoni* infection, suggesting that immune regulation may modulate the phenotype of these cells as egg burden is increased. Development of an egg challenge model in the intestine will allow comprehensive functional characterisation of the intestinal DC subsets, and enable us to study how the different subsets contribute to immune activation following egg deposition.

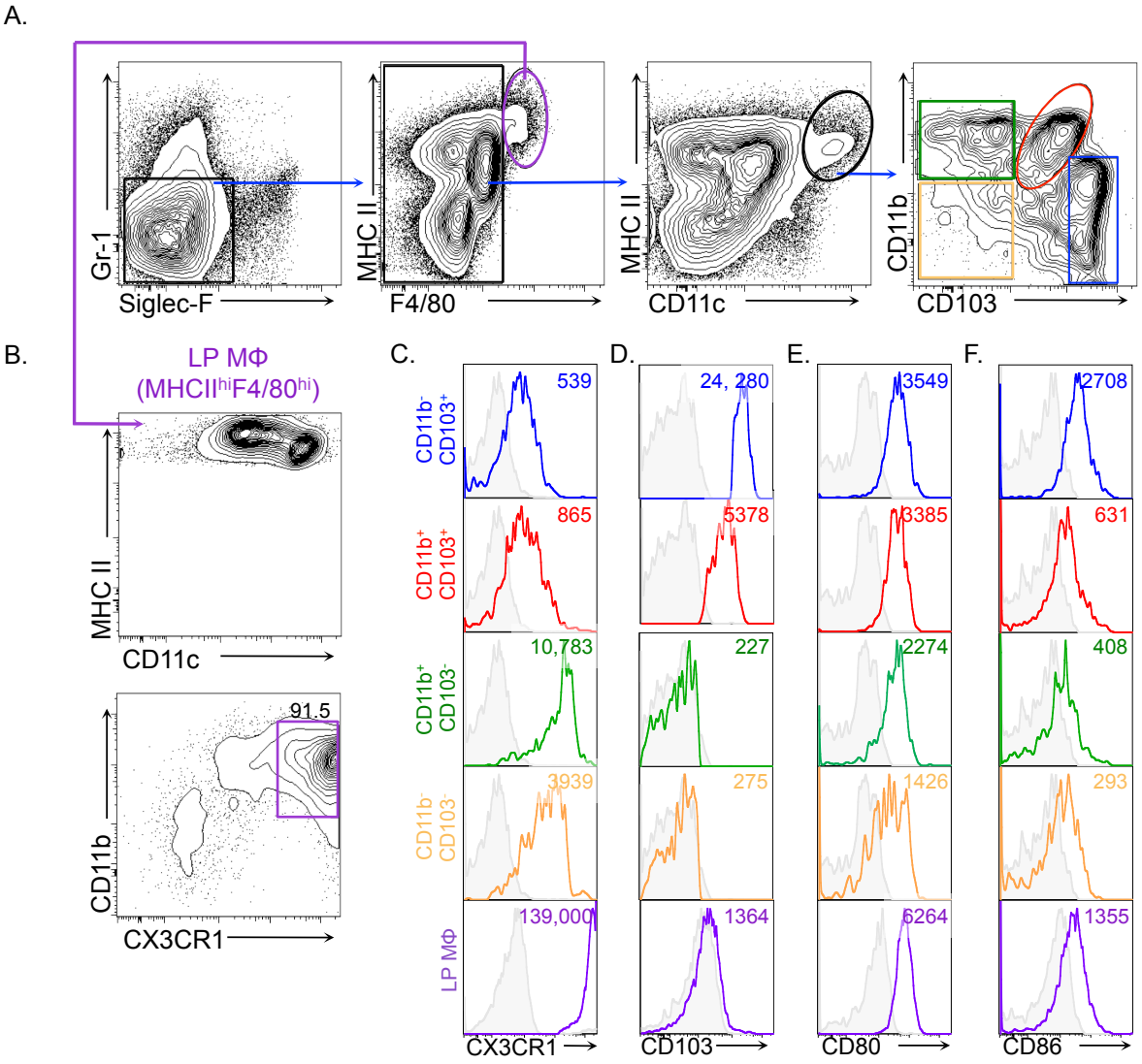

Figure 6.1 Myeloid populations in the lamina propria of the small intestine in the steady-state.
Identification of F4/80^{+} and F4/80^{-} MHC II^{hi} cell populations in the SI LP by flow cytometry, gating on live-singlet CD45^{+} cells (A). Eosinophils (Siglec-F^{+}), inflammatory monocytes (Gr1^{mid}) and neutrophils (Gr-1^{hi}) are excluded. Within the F4/80^{-} gate, the MHC II^{hi} CD11c^{+} LP DCs are further subdivided into 4 sub-populations according to their expression of CD103 and CD11b (A). F4/80^{+} MHC II^{hi} LP MΦ expression of CD11c, CD11b and CX3CR1 in a CX3CR1-eGFP mouse (B). Expression of CX3CR1 (C), CD103 (D) and the co-stimulatory molecules CD80 (E) and CD86 (F) on the LP DC subpopulations and on LP MΦ, gated as described. Grey-shaded histogram is C57BL/6 mouse (non-GFP, C) or isotype control (D-F). Fluorescence minus one (FMO) controls for all DC subsets are the same due to low numbers of cells collected (C-F). Data representative of greater than 5 experiments.

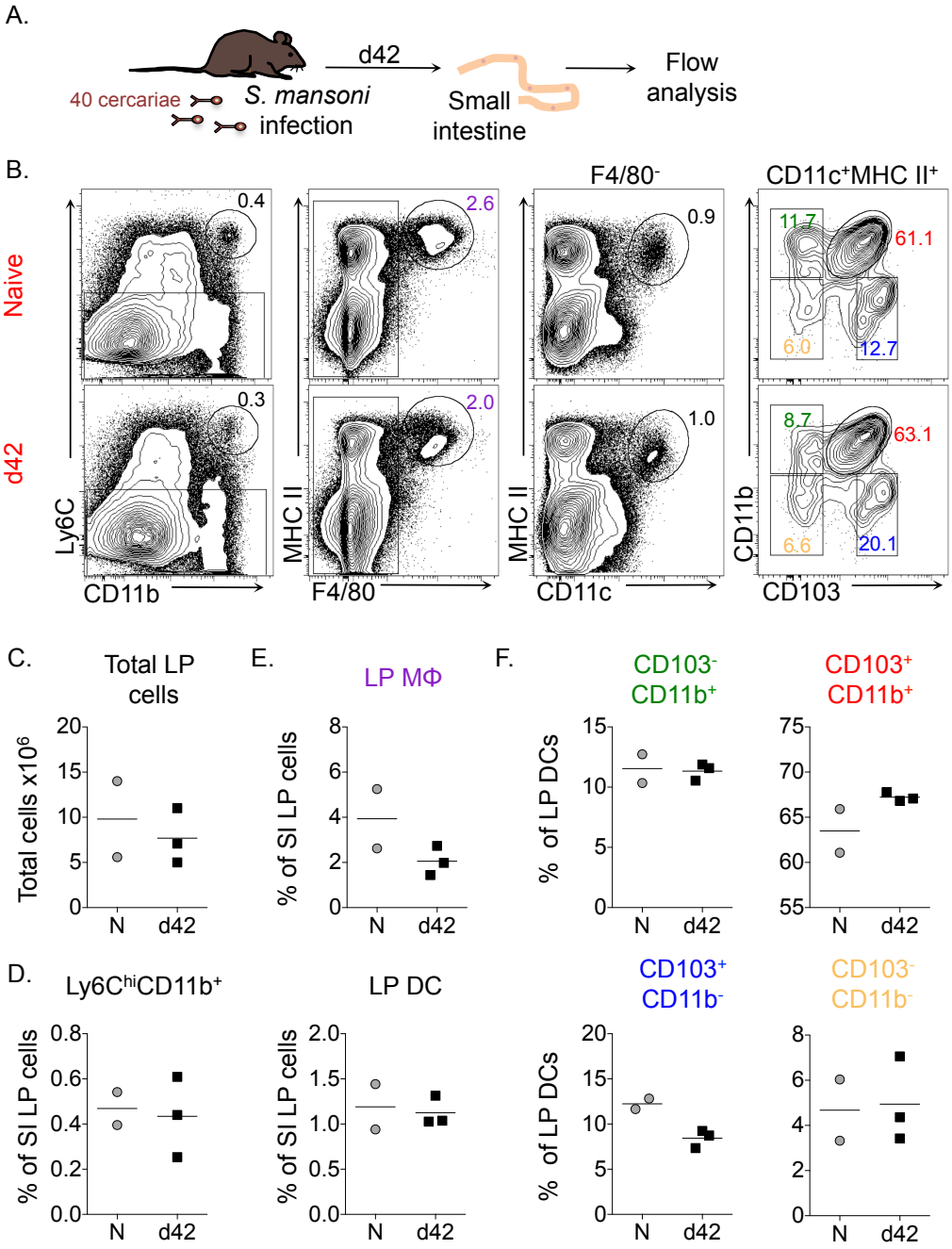

Figure 6.2 The proportions of LP MΦ and DC subsets in the small intestine are unaffected at d42 of *S. mansoni* infection.
SI LP cells were purified from naïve or d42 *S. mansoni* infected mice and live-singlet Ly6C$^-$ cells analysed by flow cytometry for characterisation of F4/80$^+$ MHC II^{hi} and F4/80$^-$ MHC II$^+$ CD11c$^+$ cell populations (B), high SSC cells were excluded from this analysis. CD11c$^+$ cell populations were further characterised by their expression of CD103 and CD11b. The proportions of F4/80$^+$ MHC II^{hi} (LP MΦ) and F4/80$^-$ MHC II$^+$ CD11c$^+$ (LP DC) were calculated as a percentage of total intact cells (C). The proportion of the different LP DC populations, defined by CD103 and CD11b expression, were calculated as a percentage of the total LP DCs in that sample (D).

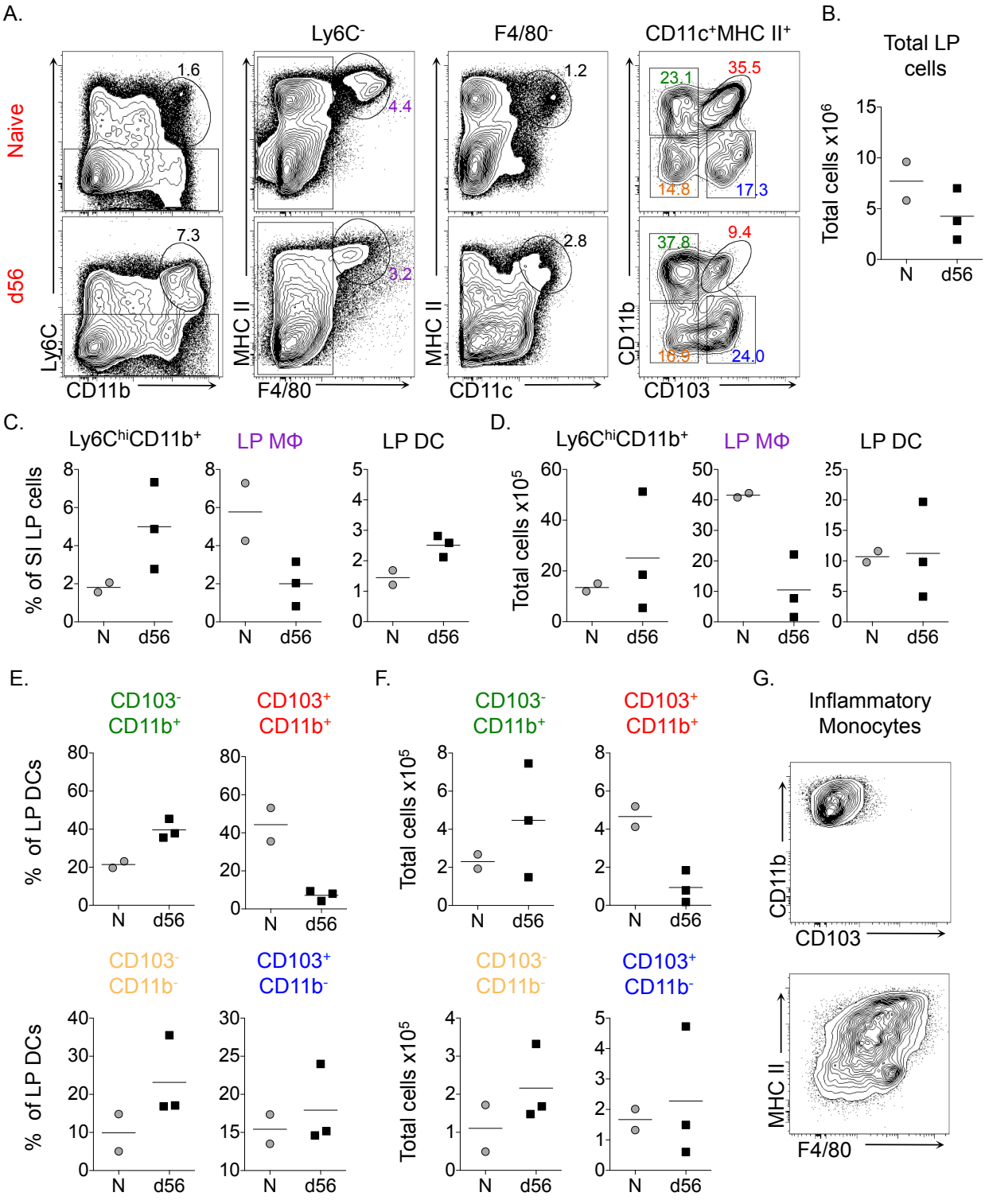
A.
Ly6C-
F4/80-
CD11c+MHC II+
Naive
d56
1.6
7.3
4.4
3.2
1.2
2.8
23.1
35.5
14.8
17.3
37.8
9.4
16.9
24.0
Ly6C
CD11b
MHC II
F4/80
MHC II
CD11c
CD11b
CD103
B.
Total LP cells
Total cells x10^6
N
d56
C.
Ly6ChiCD11b+
LP MΦ
LP DC
% of SI LP cells
D.
Total cells x10^5
E.
CD103- CD11b+
CD103+ CD11b+
CD103- CD11b-
CD103+ CD11b-
% of LP DCs
F.
Total cells x10^5
G.
Inflammatory Monocytes
CD11b
CD103
MHC II
F4/80

Figure 6.3 By d56 of infection, there is an influx of Ly6C^{hi} cells into the SI LP and changes in the make up of the DC populations.
SI LP cells were purified from naïve or d56 *S. mansoni* infected mice and live-singlet Ly6C^{-} cells analysed by flow cytometry for characterisation of F4/80^{+} MHC II^{hi} and F4/80^{-} MHC II^{+} CD11c^{+} cell populations (A), high SSC cells were excluded from this analysis. CD11c^{+} cell populations were further characterised by their expression of CD103 and CD11b. Total cell numbers from processed intestine samples from naïve (N) and *S. mansoni* infected (d56) mice (B). The proportions of Ly6C^{+}CD11b^{+} (inflammatory monocytes), F4/80^{+} MHC II^{hi} (LP MΦ) and F4/80^{-} MHC II^{+} CD11c^{+} (LP DC) were calculated as a percentage of total intact cells (C). The proportion of the different LP DC populations, defined by CD103 and CD11b expression, were calculated as a percentage of the total LP DCs in that sample (E). The number of cells from each population was calculated using the total cell numbers and the percentages calculated from flow analysis (D, F). Further characterisation of the markers expressed by the inflammatory monocyte population in the LP of an infected animal (G). Data representative of 2 mice (naïve) and 3 mice (d56 infected). Data representative of 2 experiments.

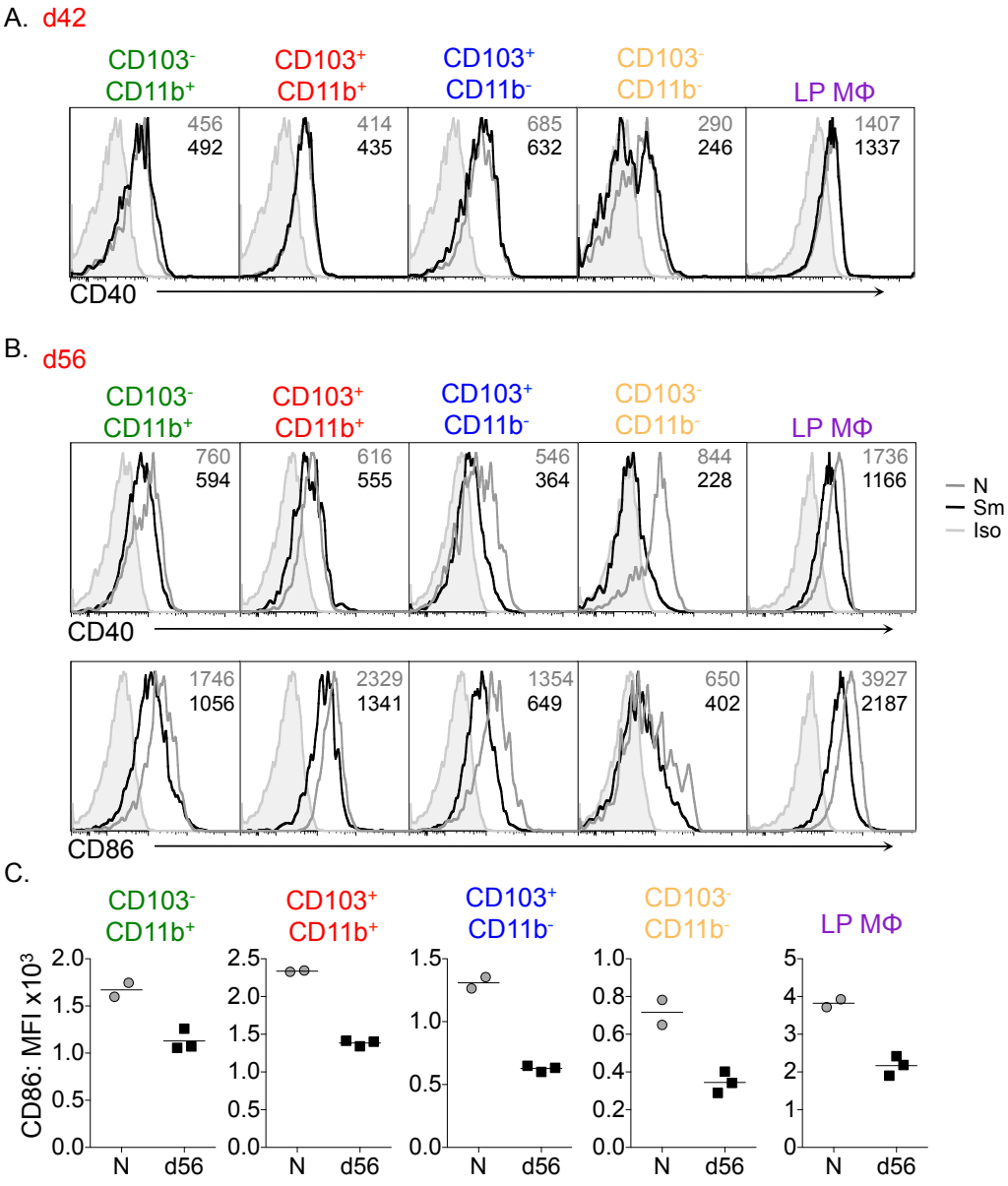

Figure 6.4 SI LP APCs downregulate their expression of co-stimulatory markers at later stages of *S. mansoni* infection.
SI LP cells were purified from naïve, d42 (A) or d56 (B-C) *S. mansoni* infected mice and live-singlet Ly6C$^-$ LP DC and LP MΦ populations analysed by flow cytometry for their expression of co-stimulatory molecules, CD40 at d42 (A), CD40 and CD86 at d56 (B-C). Values on histograms are gMFI for co-stimulatory expression (A-B). Grey-shaded histogram is isotype control (A-B). FMO controls for all DC subsets are the same due to low numbers of cells collected (A-B). GMFI was calculated for CD86 expression on naïve and d56 LP DCs and MΦ (C). Data representative of 2 mice (naïve) and 3 mice (d42/56 infected). Data representative of 2 experiments per time point.

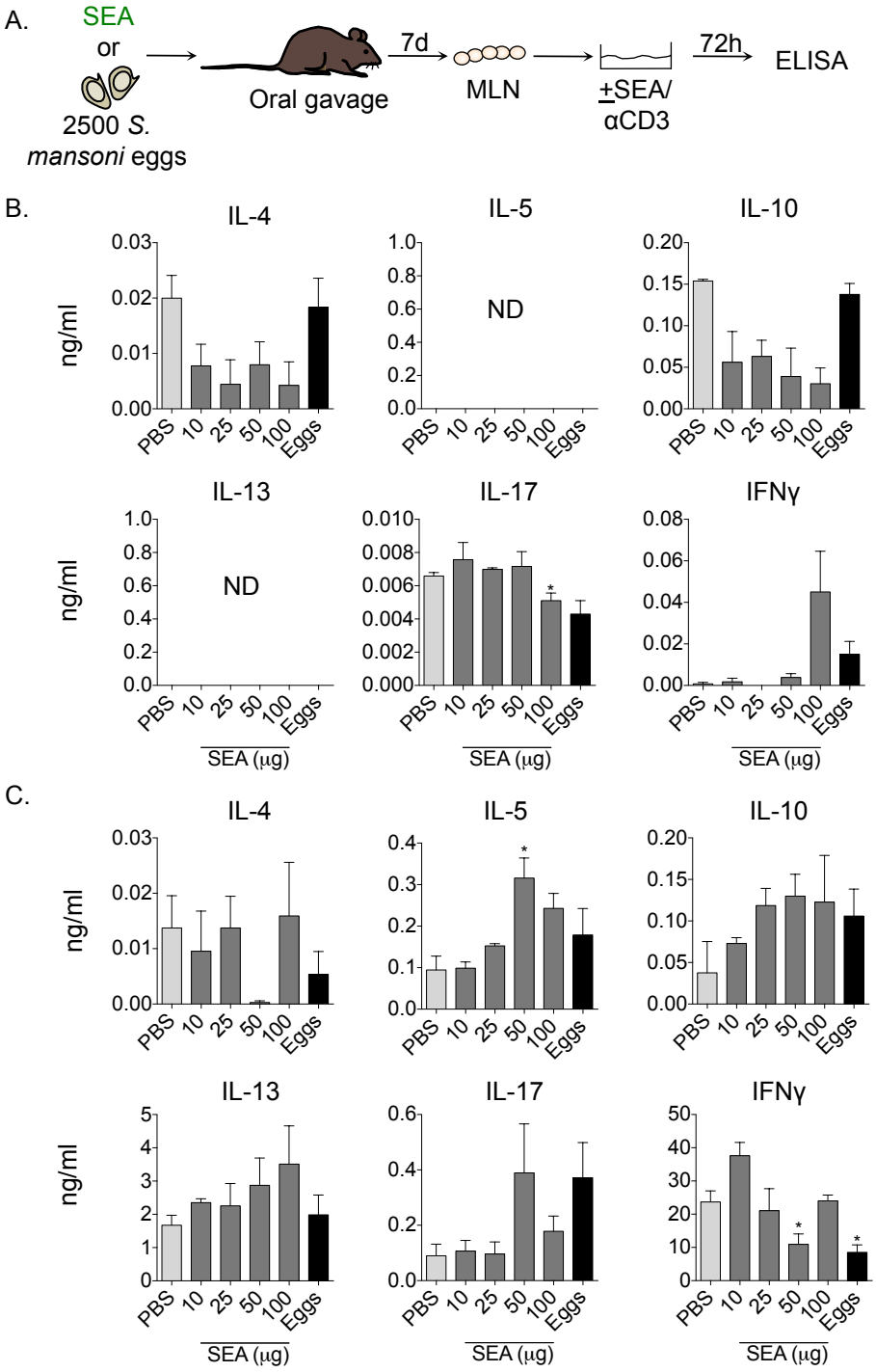

Figure 6.5 Oral administration of SEA or *S. mansoni* eggs has a low level effect on T cell cytokine responses in the MLN.
PBS, SEA (10, 25, 50, 100μg) or *S. mansoni* eggs were administered by oral gavage. 7d later, mLN were harvested and stimulated with SEA or anti-CD3. Cell supernatants were harvested 72h later for analysis of cytokine production by ELISA. SEA-specific recall response (B) and αCD3-stimulated response (C), medium background subtracted. Data from 1 experiment, 3 mice per group. *P<0.05, statistical analysis is compared to PBS cytokine level. ND – None detected.

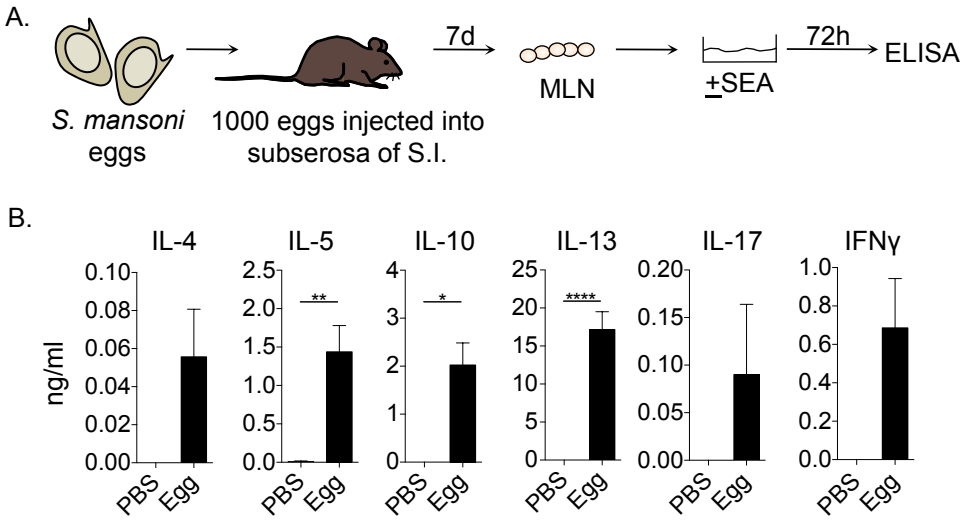

Figure 6.6 Subserosal egg injection into the small intestine leads to Th2 responses in the MLN.
Following exposure of the small intestine under anaesthetic, PBS or *S. mansoni* eggs were injected at several points along the length of the small intestine. 7d after injection, MLN were harvested and cells restimulated with SEA. Cell supernatants were collected 72h later for cytokine analysis by ELISA. Data representative of 4 experiments, 3-4 mice per group. Medium background subtracted. *P<0.05, **P<0.01, ****P<0.0001.

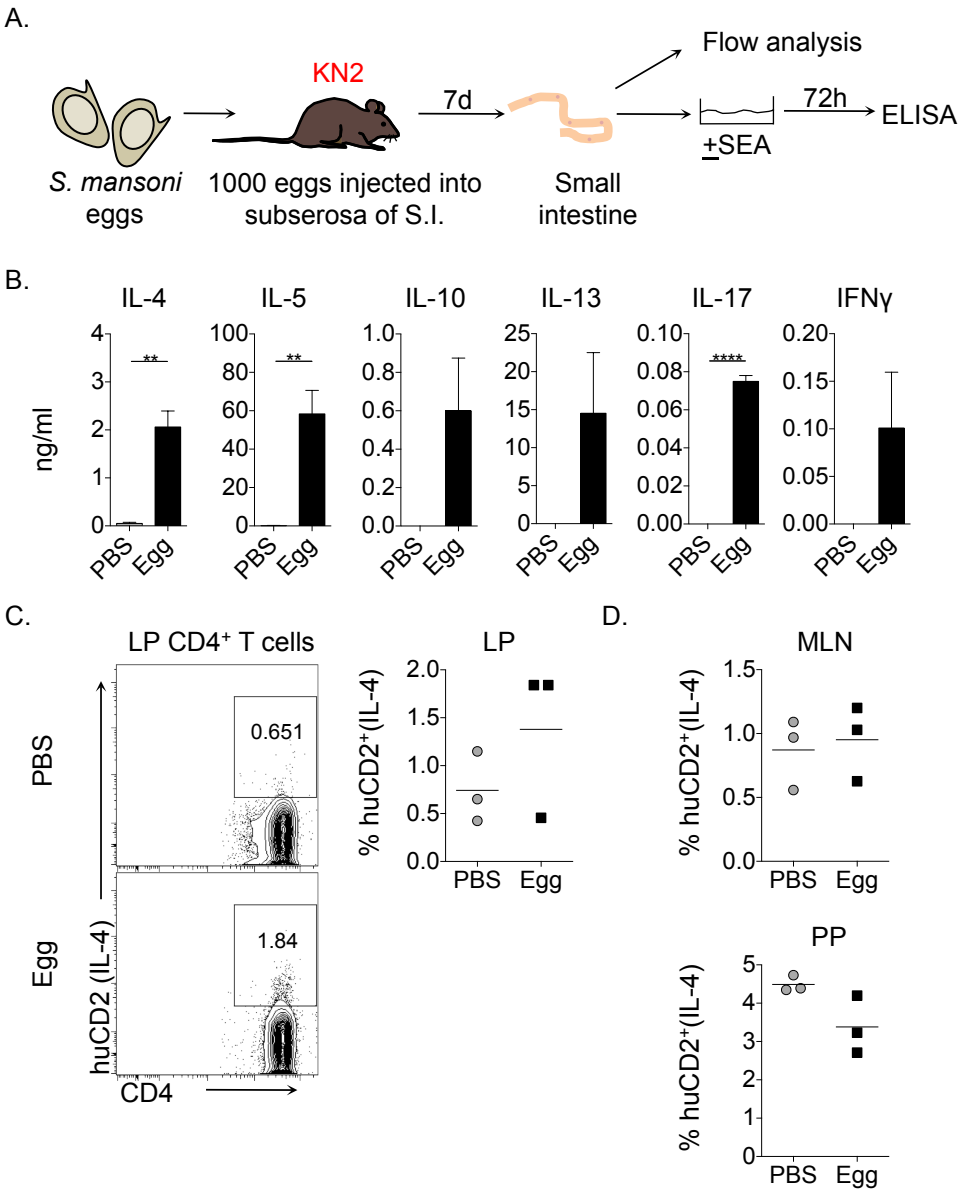

Figure 6.7 Subserosal egg injection into the small intestine leads to Th2 responses in the lamina propria.
Following exposure of the small intestine, under general anaesthetic, PBS or *S. mansoni* eggs were injected at several points along the length of the small intestine. Small intestines were harvested 7d after injection, processed to purify PP and LP cells. Cells were restimulated with SEA and supernatants were collected 72h later for cytokine analysis by ELISA (B). Cells were stained for flow cytometry, and the presence of huCD2$^+$ CD4$^+$ T cells was analysed in the LP (C), MLN & PP (D). Gated on CD45$^+$TCRβ$^+$CD4$^+$ live singlets (C-D). Data representative of 1 (C-D) or 2 experiments (B), 3-4 mice per group. Medium background subtracted (B). *P<0.05, **P<0.01, ****P<0.0001.

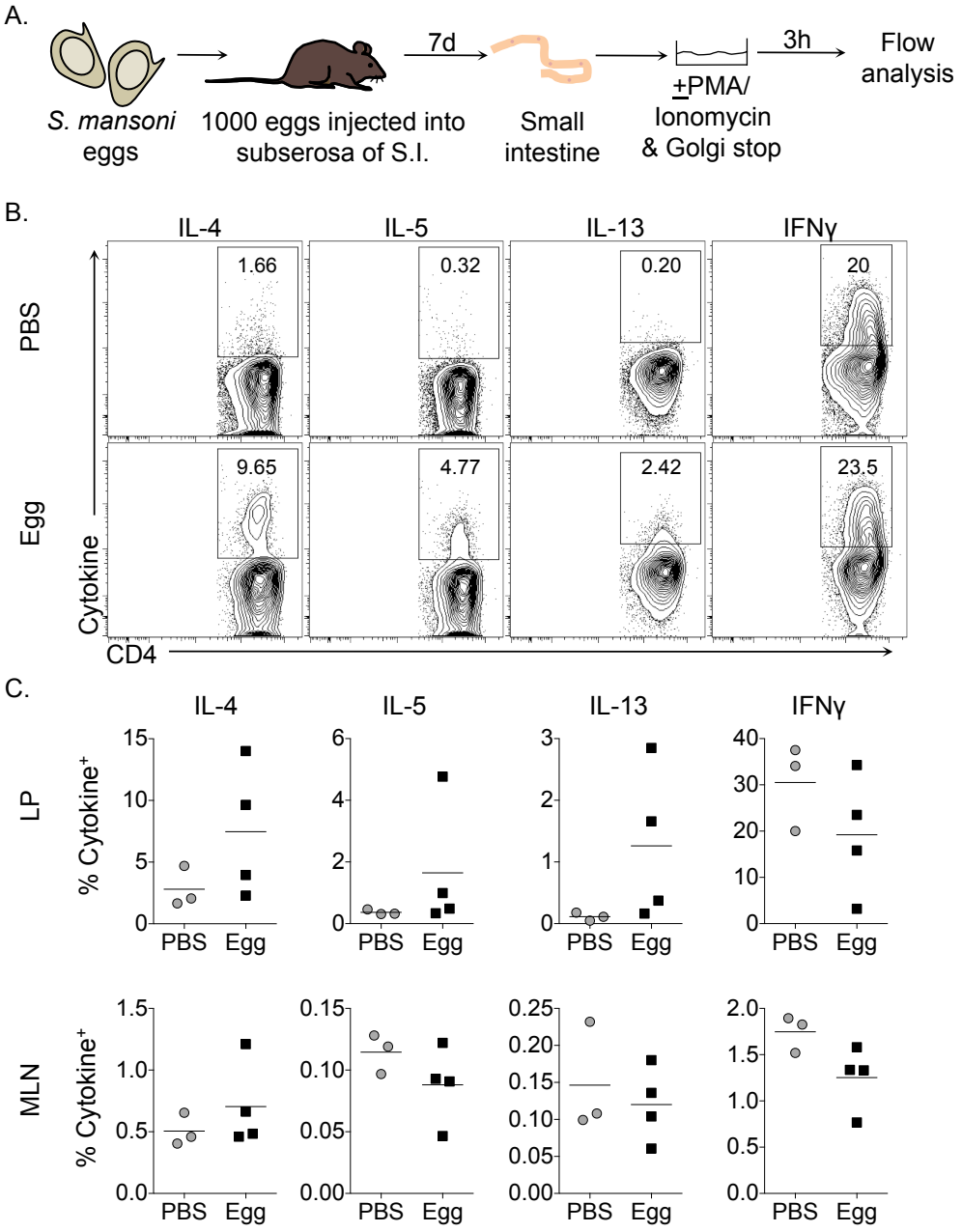

Figure 6.8 Successful subserosal egg injection into the small intestine leads to Th2 cytokine production by CD4⁺ T cells in the lamina propria.
7d after subserosal egg injection, small intestines and MLN were harvested, purified LP and MLN cells were stimulated with PMA/ Ionomycin plus Golgi stop. Cells were then stained and analysed for cytokine production by flow cytometry (B), and the percentage of cytokine⁺ CD4⁺ T cells calculated (C). Gated on live-singlet TCRβ⁺CD4⁺ cells (B). A 'successful' injection was defined by the proportion of TCRβ⁺CD4⁺ cells expressing high levels of the Th2 cytokines, IL-4, IL-5 and IL-13 (B). Data from 1 experiment, 3-4 mice per group.

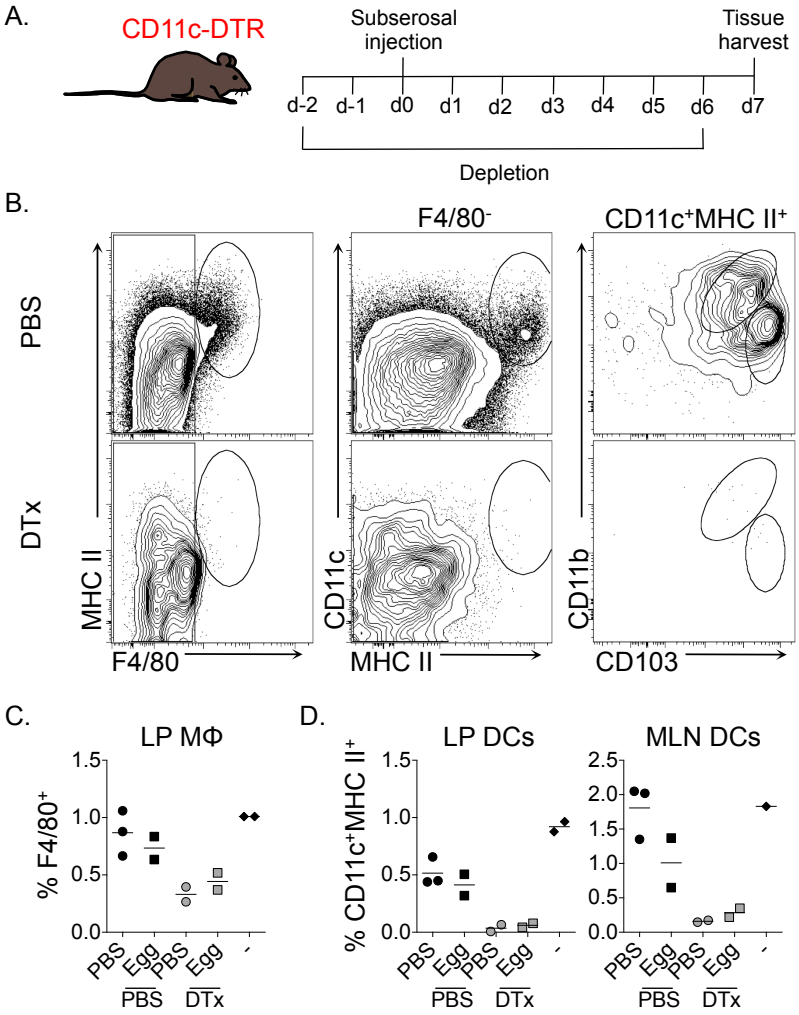

Figure 6.9 DTx treatment of CD11c-DTR mice leads to depletion of CD11c$^+$ cells in the MLN and the LP of the small intestine of PBS and egg-injected animals.
Mice were injected with PBS or DTx i.p. for 2d prior, and for 6d after, subserosal egg injection. 7d after subserosal egg injection, small intestines and MLN were harvested, purified LP and MLN cells were stained and analysed by flow cytometry. Example gating in the LP of PBS- and DTx-treated animals (B). Gated on live-singlet CD45$^+$ cells, eosinophils (SiglecF$^+$), neutrophils (Ly6G$^+$) and inflammatory monocytes (CD11b$^+$Ly6C^{hi}) are excluded from these plots. Percentages of macrophages (MΦ, F4/80$^+$) in the LP (C), and DC (CD11c$^+$MHC II$^+$) percentages in the LP and MLN (D) were calculated. (-) represents C57 BL/6 untreated mice. Data from 1 experiment, 2-3 mice per group. *P<0.05

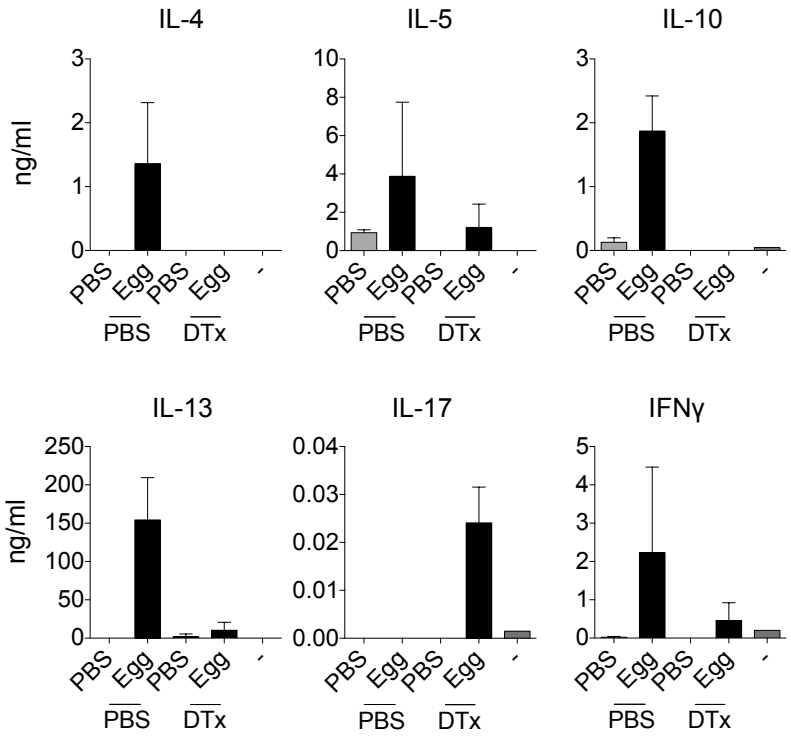

Figure 6.10 DTx treatment of CD11c-DTR mice greatly reduces the Th2 response in the mLN following subserosal egg injection.

Mice were injected with PBS or DTx i.p. for 2d prior, and for 6d after, subserosal egg injection. 7d after egg injection, mLN were harvested, and mLN cells were restimulatd with SEA. 72h later cell supernatants were collected and analysed for cytokine secretion by ELISA. Data from 1 experiment, 2-3 mice per group.

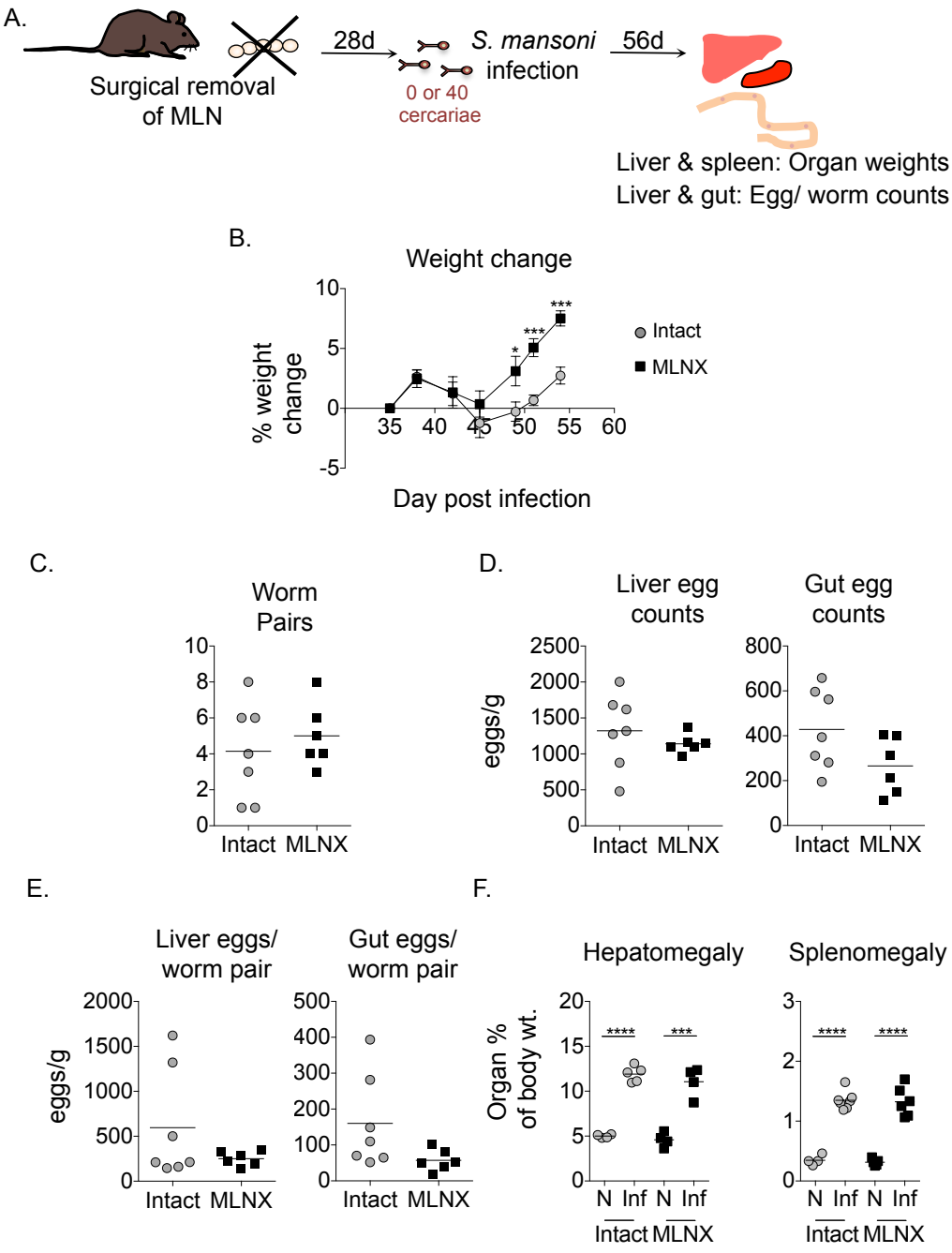

Figure 6.11 *S. mansoni*-infected mesenteric lymphadenectomy mice show similar parasitology and pathology in comparison to their intact counterparts.
MLNX mice underwent surgical removal of the mesenteric lymph nodes. 28d after surgery, a proportion of MLNX and intact animals were infected with 40 *S. mansoni* cercariae. Mouse weights were recorded from d35 of infection and weight change calculated over the following 14 days (B). On d56 of infection, animals were perfused via the heart under terminal anaesthesia. Body and organ weights were recorded, and liver and intestine samples taken for overnight digestion for egg counts (D). Worm counts were performed on perfusate (C). Eggs per worm pair were calculated from these counts (E). Splenomegaly and hepatomegaly were assessed by determining spleen and liver (F) size in relation to total body weight for uninfected and infected intact and MLNX mice. 5 mice (naïve) or 6-7 mice (infected groups). Data from 1 experiment.

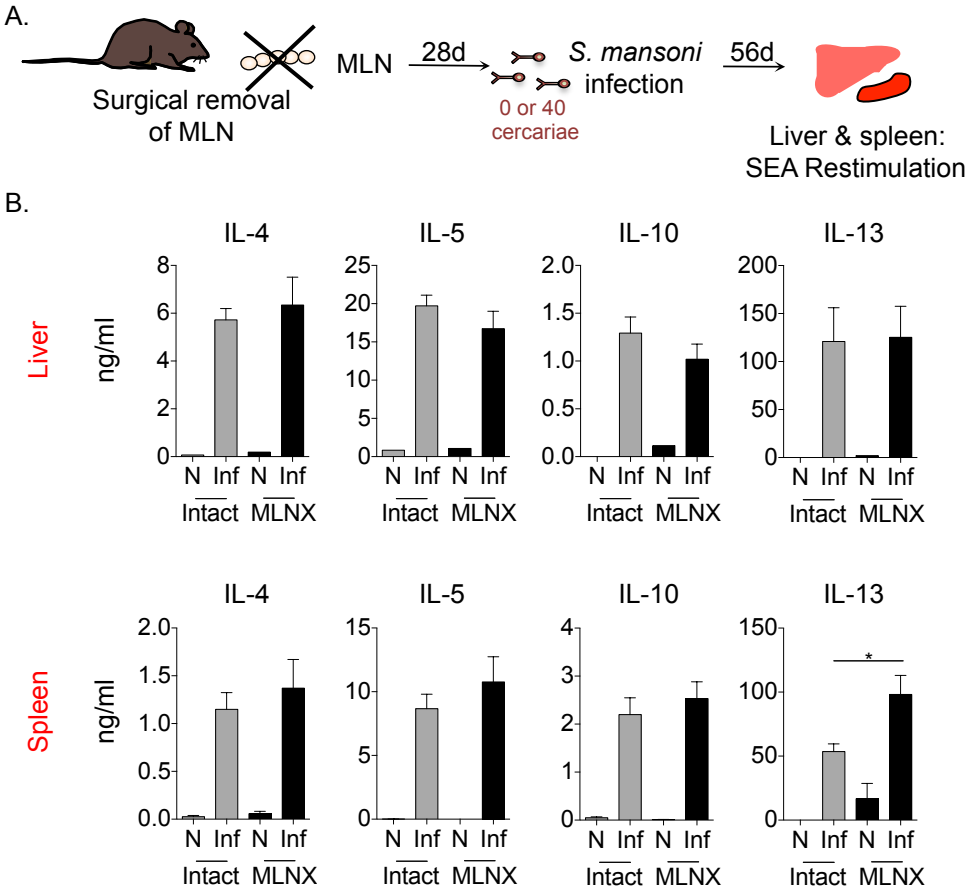

Figure 6.12 Th2 cytokine responses are unaffected in *S. mansoni*-infected mice lacking MLN.
MLNX mice underwent surgical removal of the mesenteric lymph nodes (MLNX). 28d after surgery, a proportion of MLNX and intact animals were infected with 40 *S. mansoni* cercariae. On d56 of infection, livers and spleens were harvested and cells restimulated with SEA. After 72h of culture, cell supernatants were harvested and analysed for cytokine secretion. 5 mice (naïve) or 6-7 mice (infected groups). Medium background subtracted. Data from 1 experiment. *P<0.05.

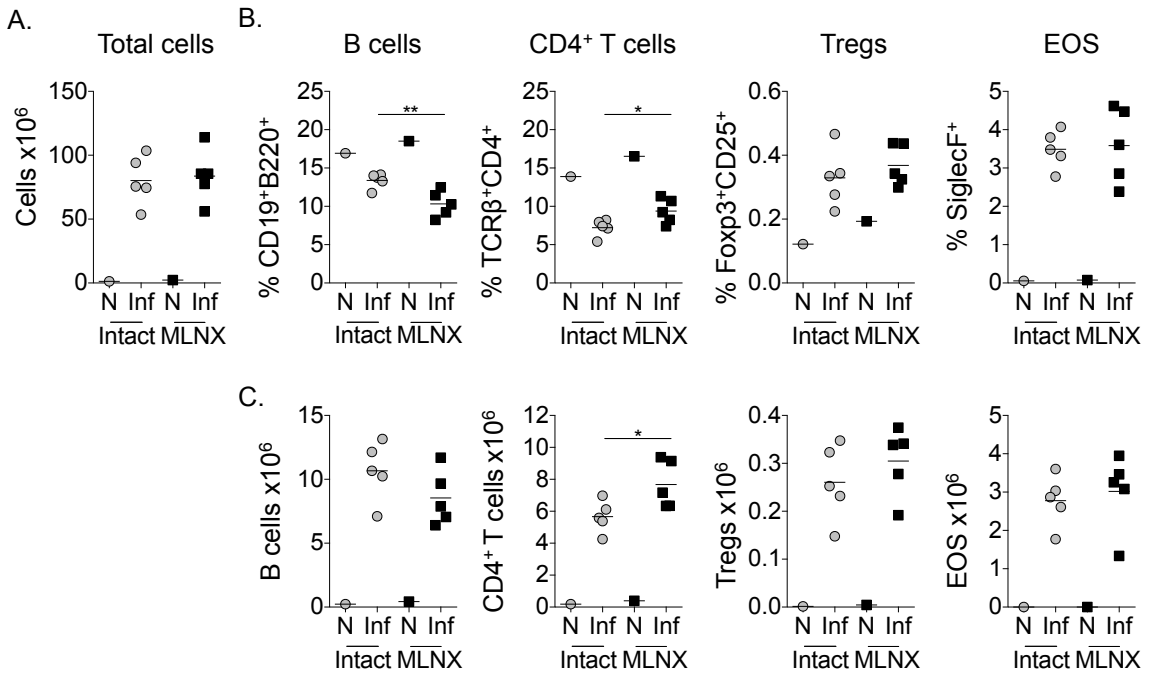

Figure 6.13 MLNX has a minor impact on lymphocyte populations in the liver of *S. mansoni* infected mice.

On d56 of infection, livers were harvested and prepared for FACS analysis. After processing, total cell numbers were calculated per sample (A). Cells were gated on live, intact-singlets. Proportions of cell populations were calculated as a percentage of intact live-singlets, using event counts for each population (B). Actual numbers of cell populations was calculated from cell counts using percentages from flow analysis (C). Livers from 5 mice pooled (naïve) or 5 individual livers (infected groups). Data from 1 experiment. *P<0.05, **P<0.01.

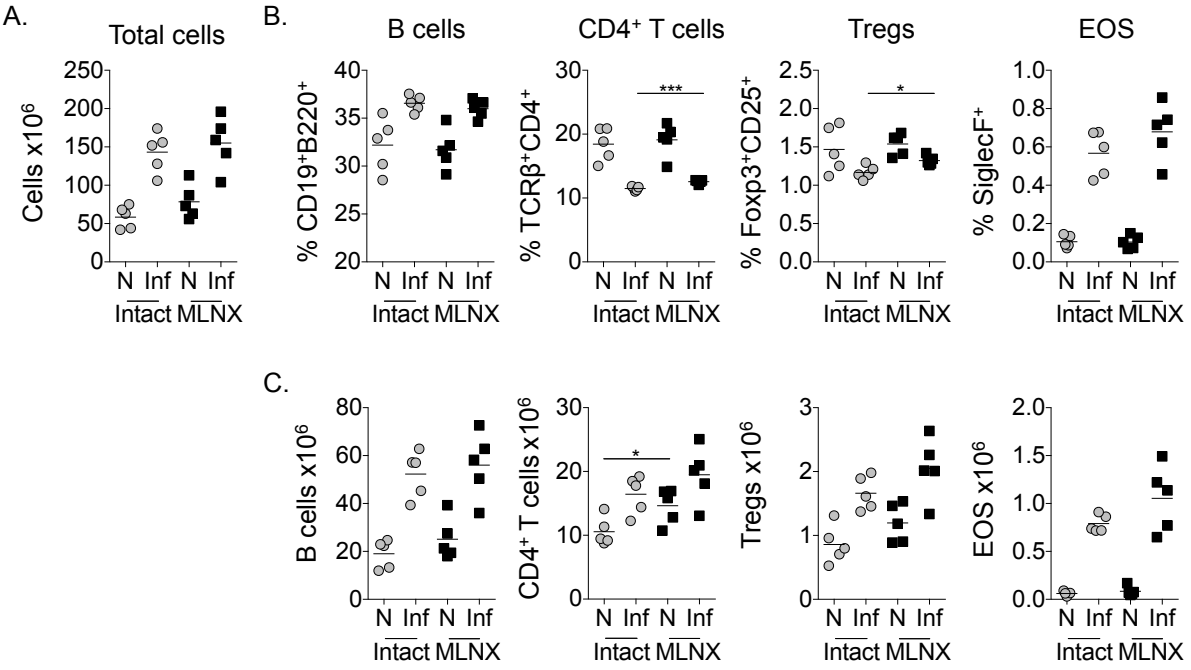

Figure 6.14 MLNX has a minor impact on lymphocyte populations in the spleen of *S. mansoni* infected mice.
On d56 of infection, spleens were harvested and prepared for FACS analysis. After processing, total cell numbers were calculated per sample (A). Cells were gated on live, intact-singlets. Proportions of cell populations were calculated as a percentage of intact live-singlets, using event counts for each population (B). Actual numbers of cell populations was calculated from cell counts using percentages from flow analysis (C). 5 mice per group. Data from 1 experiment. *P<0.05, ***P<0.001.

7.0 GENERAL DISCUSSION

At the outset of the work detailed in this thesis, whilst there was some understanding of how DCs respond to helminth Ag, much remained unexplained about the complexity of DC activation and function in the context of the Th2 response. By systematically assessing the phenotype and function of Flt3-L-dependent DCs to *S. mansoni* egg Ags, with a focus on the role of IFN-I in this context, then beginning to link the results generated in those experimental systems to relevant *in vivo* settings, including egg or Ag injection and murine models of helminth infection, we have advanced our understanding of a number of key areas of DC orchestration of Type 2 immunity. Moreover, we have provided a platform for future work expanding on this, which will delineate the importance of IFN-I in Th2 settings and the involvement of intestinal DCs in immune activation against helminths.

Different DC subsets have distinct roles in the management of immune activation, and it is becoming increasingly clear that this is particularly the case in the context of Th2 responses. We have demonstrated for the first time that Flt3-L dependent DCs respond to *S. mansoni* soluble egg Ags in a subset-specific manner.

7.1 Surface activation of Flt3-L dependent DCs by SEA

Previous studies using GMDCs have demonstrated that these cells are not phenotypically activated by exposure to SEA (MacDonald *et al.*, 2001; Perona-Wright *et al.*, 2006a). However, we have found that Flt3-L dependent cDCs displayed a level of surface activation in response to SEA, upregulating expression of MHC II and co-stimulatory molecules (Fig. 3.2). However, SEA elicited an intermediate activation phenotype, as expression levels of co-stimulatory molecules were somewhat lower than those of classically activated DCs exposed to the bacteria St.

Whilst Flt3-L generated pDCs did become activated by SEA, their level of surface activation was several orders of magnitude below that displayed by the Flt3-L dependent cDC subsets. This suggests that pDCs would not be capable of priming SEA-specific T cells, a hypothesis that is supported by other recent work in the lab that has shown that pDCs purified from the livers of *S. mansoni* infected mice can only initiate proliferation of pre-activated, or memory, T cells (Rachel Lundie *et al.*, manuscript submitted).

7.2 SEA-specific cytokine production by Flt3-L-dependent DCs

GMDCs do not produce cytokines in response to SEA (MacDonald *et al.*, 2001; Perona-Wright *et al.*, 2006a), indeed, limited cytokine production is a hallmark of the response of DCs to helminths in general, not only *S. mansoni* (Balic *et al.*, 2004; MacDonald and Maizels, 2008; Segura *et al.*, 2007; Whelan *et al.*, 2000). This is likely to be an evolved response that favours both the host and the parasite (Dunne and Cooke, 2005; Everts *et al.*, 2010b). In natural infection, helminths may persist within the host for many years, meaning that the host is continuously exposed to parasite Ags for an extended period. By avoiding excessive and prolonged immune activation - which would be the likely outcome if DCs did produce large quantities of cytokines in response to helminth Ag - the host is protected from damaging immunopathology. A moderate response also benefits the parasite, as muted effector activity, alongside immune regulation, seems to favour helminth survival and fecundity (Dunne and Cooke, 2005; Everts *et al.*, 2010b). Persistance of the parasite is dependent on host survival, thus helminths benefit from a muted immune response that does not cause severe morbidity or mortality. This may also explain why helminth Ags can act to directly inhibit classical DC activation by bacteria and defined TLR ligands (Carvalho *et al.*, 2009) – dampening any resultant immune activation against bystander pathogens that could impact on parasite survival.

We have demonstrated that FLDCs produced only very low-levels of the innate cytokines commonly associated with classical DC activation – for example IL-12, IL-6, TNFα – following exposure to SEA. Unexpectedly, however, an SEA-specific IFN-I signature was identified, with the $CD24^+$ cDCs (the *in vitro*-generated $CD8\alpha^+$ cDC equivalents) alone secreting SEA-specific IFN-I. This surprising finding raises a number of fundamental questions: Why is it that $CD24^+$ cDCs uniquely produced IFN-I following exposure to SEA? What are the ligands and receptors that orchestrate the IFN-I response of DCs to SEA? Why would DCs produce IFN-I in a Th2 setting, when this cytokine is normally more clearly associated with Th1/17 pathogens?

7.2.1 Why is the SEA IFN-I response not seen in all DC subsets? SEA sensing and signalling pathways in the IFN-I response

The most likely explanation for why only $CD24^+$ cDCs produced IFN-I in response to SEA is that only this subset expresses the PRR(s) and/or signalling molecules necessary to activate this response. Thus in the process of investigating why the IFN-I response is unique to $CD24^+$ cDCs, we hope to delineate the PRRs that orchestrate this response. In beginning to address this, we have shown that SEA-specific IFN-I production by FLDCs is wholly dependent on the adaptor protein TRIF, whilst optimal IFN-I induction also requires CD205. This CLR is selectively expressed by $CD8\alpha^+$ cDCs *in vivo* (Henri *et al.*, 2001; Vremec *et al.*, 2000), and is most likely restricted to $CD24^+$ *in vitro*-generated cDCs.

Given that the IFN-I response to SEA appears to be unique to $CD24^+$ cDCs and is dependent on TRIF, we hypothesise that TLR3 is involved, as amongst FLDCs, this receptor is expressed uniquely by the $CD24^+$ subset, and is not present at high levels in GMDCs (Jelinek *et al.*, 2011; Naik *et al.*, 2005). However, we were not able to detect any significant activation of a TLR3 reporter cell line by SEA (Section 3.2.8, Fig. 3.9). This discrepancy may be explained by the involvement of a distinct TRIF-dependent PRR, such as a cytosolic helicase sensor that is involved in nucleic acid sensing (Zhang *et al.*, 2011a). Alternatively, CD205 (and potentially other CLRs) may be essential intermediaries that are required for SEA-specific IFN-I induction (Fig. 7.1).

CLRs, such as CD205, may enable or enhance uptake of bioactive SEA ligands that may be involved in the generation of further immunostimulatory Ags. It has already been shown that the ability of omega-1, a glycoprotein present in SEA, to activate Th2 responses is dependent on its RNase function (Everts *et al.*, 2012; Steinfelder *et al.*, 2009). MR mediates omega-1 uptake and in the absence of DC MR expression, omega-1 fails to elicit Th2 responses (Everts *et al.*, 2012). This raises the interesting question of whether omega-1 – and other SEA glycoproteins – can generate nucleic acid ligands that stimulate the IFN-I response, following uptake by CLRs.

Another CLR, Dectin-1, can initiate IFN-I production via a distinct pathway (Del Fresno *et al.*, 2013), thus it should not be ruled out that CD205 ligation by SEA activates IFN-I production directly. It is also possible that CD205 and a TRIF-dependent receptor

synergise in the activation of SEA IFN-I, as has previously been shown to occur when Dectin-1 and MyD88-dependent TLRs are activated simultaneously (Dennehy *et al.*, 2008). Given that SEA is made up of a diverse and complex mixture of different Ags – including glyocproteins, lipids, carbohydrates and nucleic acids (Aksoy *et al.*, 2005; Ashton *et al.*, 2001; Dunne *et al.*, 1991; Mathieson and Wilson, 2010; Meevissen *et al.*, 2011; Verjovski-Almeida *et al.*, 2003) - it is feasible that one SEA Ag activates CD205, whilst another stimulates TRIF-dependent receptor(s), leading to SEA-specific IFN-I production (Fig. 7.1).

It is known that many Ags in SEA, including the most potent immune activators, are CLR ligands (Meevissen *et al.*, 2011), and it had seemed that TLRs were not essential for recognition of SEA by DCs or for immune activation during *S. mansoni* infection (Jankovic *et al.*, 2004; Layland *et al.*, 2005; Vanhoutte *et al.*, 2008). However, our findings suggest that there may be synergy of different PRR pathways for optimal immune activation against *S. mansoni* egg Ags. The discovery that MyD88 can negatively regulate the SEA-specific IFN-I response is further evidence that there is a complex interplay of receptor-signalling pathways in the recognition of these Ags.

Our finding that the CLR CD205 is involved in the induction of SEA-specific IFN-I suggests that Ag(s) involved in this response include glycoproteins or carbohydrate ligands. This is in agreement with other studies that have identified a number of CLRs capable of recognising components of SEA (Everts *et al.*, 2012; Meevissen *et al.*, 2012; Meevissen *et al.*, 2011; van Die *et al.*, 2003). The involvement of TRIF suggests that nucleic acid ligands may also be involved in the activation of this response, as TRIF-dependent TLR3 and the cytosolic helicase nucleic acid sensors both recognise dsRNA (Alexopoulou *et al.*, 2001; Zhang *et al.*, 2011a). However, TRIF is also involved in mediating signals following TLR4 activation, which recognises a range of ligands, including LPS, and the carbohydrate ligand LNFPIII present in SEA (Thomas *et al.*, 2003). Thus, there are as many potential ligands involved in the IFN-I response as potential PRR pathways.

Further work is required to identify the specific ligands and receptors that orchestrate the IFN-I response, and to address their importance for immune activation against *S. mansoni*. Thus far we have performed a number of studies using BMDCs deficient in specific PRRs

or adaptor proteins to establish their role in IFN-I induction. In the future we would like to assess the role of the TRIF-dependent TLRs using TLR3 and TLR4 deficient cells. However, with our hypothesis that a number of different receptors may be involved, microarray analysis of the gene expression changes of FLDC subsets following exposure to SEA is a key experiment. This will enable us to perform network analysis to identify the different signalling pathways up or downregulated in response to SEA, as well as potentially identifying the specific TFs involved in IFN-I activation, and other ISGs upregulated in response to SEA. This will give us a much greater understanding of the SEA-specific IFN-I signature and the potential importance of this for Th2 polarisation by Flt3-L dependent DCs.

At the moment we have a number of different candidate pathways and TFs that may be involved in IFN-I induction or activated downstream of IFNAR. Whilst the transcription of some of these factors may be upregulated in DCs exposed to SEA, the expression of many will remain unchanged. Instead, they will become activated by phosphorylation. Phosphoproteomic analysis would identify the signalling molecules and TFs that are phosphorylated following exposure to SEA, providing a fuller picture of the cellular changes in FLDCs that may be involved in SEA sensing. This technique has already been used to characterise the phosphorylation events in T cells following TCR ligation (Navarro *et al.*, 2011), but there have been no published studies in DCs as yet.

7.2.2 The importance of SEA IFN-I for DC function in Th2 settings

The discovery of an SEA-specific cytokine signature is a novel and exciting finding in the world of Th2, given that no such signal has previously been identified. However, it is unlikely that IFN-I is the elusive 'signal 3' required for Th2 induction, given that GMDCs readily polarise Th2 responses but fail to secrete IFN-I in response to SEA (Fig. 3.6C). In light of this, why do Flt3-L-dependent DCs produce IFN-I in response to SEA? Our hypothesis is that IFN-I acts in an autocrine manner to maximise DC functionality in response to helminth Ag. We have shown that optimal FLDC responses to SEA depended on their ability to respond to IFN-I. SEA-specific Th2 polarisation by FLDCs *in vivo* was entirely ablated in the absence of IFNAR (Fig 4.6). However, IFNAR-deficient FLDC function *in vitro* was unaffected (Fig. 4.11-Fig.4.13). This discrepancy immediately suggested to us that FLDC migration might be the key factor affected by IFNAR deficiency.

DC migration in response to helminth Ags is another major unknown in the Th2 field. Despite expressing relatively low-levels of CCR7 in response to SEA, FL-cDCs migrated effectively to the dLN. In support of our hypothesis that IFNAR is required for effective FLDC migration, CCR7 expression was reduced on IFNAR-deficient cDC subsets (Fig. 4.14). However, we are yet to show definitively that IFNAR is required for FL-cDC migration.

Intriguingly, ligation of IFNAR leads to the activation of the GTPase Rap1 (Lekmine *et al.*, 2002). This GTPase is able to activate a range of effector proteins involved in cellular processes. One such effector protein is RapL. RapL and Rap1 are crucially involved in DC adhesion and motility. In the absence of RapL, DC ability to adhere to the integrin ICAM-1 via LFA-1, is severely reduced, and DC migration to and localisation within lymphoid organs is defective (Katagiri *et al.*, 2004). Given that LFA-1 is also involved in the formation of the immunological synapse between DCs and T cells (Balkow *et al.*, 2010; Brossard *et al.*, 2005), it is likely that Rap1 effectors are also involved in this process. It is tempting to speculate that a deficiency in IFNAR function would impact on the activity of the Rap pathway, leading to defective DC migration, localisation and T cell interactions. Thus, we may have identified in IFN-I an important factor that facilitates the migration of Flt3-L dependent DCs. Further investigations of these processes will enhance our understanding of DC mobilisation and function in Th2 settings. The subserosal egg injection model, along with the development of the cannulation technique, provides an ideal tool to study the migration of resident DCs following exposure to helminth Ag, in a site that is relevant to *S. mansoni* infection. This model will allow us to further dissect the importance of IFNAR for efficient DC migration from peripheral tissues to the dLN.

7.3 The role of IFN-I in helminth-induced Th2 inflammation

We have begun to address the impact of IFN-I *in vivo* during patent murine helminth infection. Th2 cytokine levels were ablated in the MLN of *S. mansoni* and *H. polygyrus* infected IFNAR-deficient mice (Fig. 5.7C and 5.9D). Intriguingly, no dramatic reduction in the Th2 cytokine output was detected in the liver of *S. mansoni* infected IFNAR-deficient mice (Fig. 5.7B). It is not yet known whether granuloma formation or long-term host survival is affected in the absence of IFNAR, thus longitudinal studies are required to address these questions in both infection models.

Helminth infection is complex, with parasites - or their eggs - causing damage to tissues, whilst many other sites are exposed to systemic Ag. The immune response in *S. mansoni* and *H. polygyrus* infection is predominantly polarised towards Th2; however, there are also elements of Th1 and Th17 activation, as well as regulatory responses. For example, at the early stages of *S. mansoni* infection, prior to egg laying, the immune response is mixed Th1-Th2. Commencement of egg deposition stimulates a strongly Th2 dominated response, and at the chronic stages of infection, regulation comes to the fore (Pearce and MacDonald, 2002). Thus, various timepoints and tissue sites must be considered to facilitate meaningful understanding of the role of IFN-I and IFNAR during helminth infection. Looking at one stage of infection or at one specific tissue will not provide a credible or comprehensive interpretation.

Given that we have identified a dichotomy between FLDCs and GMDCs in the requirement for IFNAR in SEA-specific Th2 induction *in vivo*, it was intriguing to find that Th2 responses in *S. mansoni* mice were limited in the MLN but not the liver. It could be hypothesised that the deficiency in Th2 induction is restricted to lymphoid organs where GM-CSF plays no role in DC homeostasis (Kingston *et al.*, 2009; McKenna *et al.*, 2000), whilst Th2 responses remain unaffected in peripheral tissues, where GM-CSF is required for the maintenance of DC populations (Greter *et al.*, 2012). Further work is needed to examine the Th2 response in a broader range of tissues, in both *S. mansoni* and *H. polygyrus* infected mice, to test this hypothesis. Analysis of the phenotype and function of DCs from infected IFNAR-deficient mice is also required.

If IFN-I is involved in the maintenance of DCs in lymphoid organs, this may explain why Th2 cytokine levels were reduced in the MLN. The flaw in this hypothesis is that (GM-CSF-dependent) DCs migrating from the tissue are almost certainly involved in Th2 polarisation, so would a deficiency in LN DC function impact greatly on the development of the T cell response? It has been shown that resident DCs within the T cell zone can take up soluble Ag that enters from the afferent lymphatics, and prime early T cell responses, before Ag-loaded migrating DCs arrive in the dLN (Iezzi *et al.*, 2006; Sixt *et al.*, 2005). This has been demonstrated using fluorescently-labelled synthetic Ag (Sixt *et al.*, 2005), but also during Leishmania infection (Iezzi *et al.*, 2006). However, in both settings this phenomenon occurs very early, within 24h of Ag administration/ infection. One may expect by d42 of *S.*

mansoni infection that any early deficit in Th2 development would be overcome. It has been shown during helminth infection, that IL-4 signalling is diffuse throughout the reactive LN (Perona-Wright *et al.*, 2010), which likely sustains Th2 induction. Whether DCs are involved in the propagation of the IL-4 signal has not been addressed. However, if LN-resident DCs are involved, this response could be abrogated in IFNAR-deficient animals, if DC function in the LN is curtailed in the absence of IFN-I signalling.

Studies have suggested that during helminth infection and allergic responses, a proportion of Th2 cytokine-producing cells in the reactive LN are Tfh cells (King and Mohrs, 2009; Liang *et al.*, 2011). It has been demonstrated that during T cell priming in the MLN, Tfh and gut-homing T cells (expressing the gut-homing integrin $\alpha_4\beta_7$) are generated as separate subsets (Cucak *et al.*, 2009). The $\alpha_4\beta_7^+$ T cells, but not Tfh, rapidly exit the MLN following priming. The authors demonstrate that priming of Tfh cells is dependent on DC expression of IFNAR, as DCs require IFNAR expression in order to produce IL-6, which mediates the development of Tfh cells (Cucak *et al.*, 2009). This would fit with our finding that the Th2 response in the MLN during *S. mansoni* and *H. polygyrus* infection is selectively affected by IFNAR-deficiency. To investigate this, future experiments should examine whether Tfh cells ($CXCR5^+$ $ICOS^+$ $PD\text{-}1^+$ T cells) display a selective deficiency in Th2 cytokine production in the LNs. If Tfh responses were affected, we would also expect to see an impact on the development of humoral responses, which we are yet to address.

We have suggested that IFNAR-deficient DCs may be unable to prime Th2 responses due to defective migration. As outlined above (Section 2.2.2), ligation of IFNAR leads to the activation of the Rap pathway, which is essential for effective DC adhesion and migration to the dLN (Katagiri *et al.*, 2004). In IFNAR-deficient mice it is possible that activation of the Rap1 GTPase and its effector proteins would be abrogated, leading to defective DC mobilisation. This would certainly impact on the induction of the immune response during helminth infection.

The drawback of investigating the role of IFN-I in complete IFNAR-deficient mice is that it is difficult to pinpoint which cell type(s) require IFN-I signalling for optimal Th2 induction to occur. Although IFN-I impacts on DC phenotype and function (Ng and Gommerman, 2013), which influences T cell responses, it can also act directly on T cells (Tough, 2012),

as well as on other immune cells, including MΦs, B cells and NK cells (Cervantes-Barragan *et al.*, 2007; Kiefer *et al.*, 2012; Mizutani *et al.*, 2012). For this reason, the impact of IFNAR deficiency on the function of other cell types should also be considered.

One aspect of immune cell function that may be altered in the absence of IFNAR is the controlled movement of cells in and out of lymphoid organs. IFN-I is involved in limiting immune cell egress from LNs - activation of IFNAR on the surface of leukocytes leads to the upregulation of CD69 and the consequent downregulation of S1PR1, preventing leukocyte exit from the LN (Cyster and Schwab, 2012; Shiow *et al.*, 2006). In the absence of IFNAR, lymphocyte egress may be enhanced, resulting in a reduction in the proportion of Th2 cells that remain in the lymphoid organs. As well as being active in DCs, the Rap1 pathway is also involved in the upregulation of LFA-1 following ligation of the TCR, allowing arrest of T cells and formation of the immunological synapse with DCs, as well as effective migration (Ebisuno *et al.*, 2010; Hogg *et al.*, 2011). If the Rap1 pathway is defective in the absence of IFNAR, this will likely affect the function and activation of T cells, as well as DCs.

This does not rule out a role for DCs in this process, however, as they may act as an important source of IFN-I in this setting. Thus, it is necessary to further investigate the requirement for IFNAR on myeloid cells *and* T cells. This could be done by selective deletion of IFNAR expression on T cells or $CD11c^+$ cells, using CD4- or CD11c-cre mice crossed with IFNAR-flox animals. In the absence of these models, mixed chimeras of CD45.1 WT: CD45.2 IFNAR-deficient BM would allow us to study IFNAR-deficient DCs or T cells and sort them for functional assays, whilst all radioresistant cells would be WT. This approach would enable assessment of the activation phenotype and priming ability of IFNAR-deficient DCs from *S. mansoni* infected mice, and the investigation of IFNAR-deficient T cell cytokine production/ proliferation. Initially, it would be informative to examine the expression of CD69 and S1PR1 by different cell populations in the LN and peripheral tissues during helminth infection. It is also necessary to assess the proportion of T cells producing Th2 cytokines in the LN of IFNAR-deficient mice during infection by ICC or using cytokine reporter mice, such as KN2 mice, to assess IL-4 production (Mohrs *et al.*, 2005), and IL-4/ IL-13/ IL-10eGFP mice to measure mRNA levels of these three cytokines (Kamanaka *et al.*, 2006; Mohrs *et al.*, 2001; Neill *et al.*, 2010).

If IFNAR were required to maintain T cells in the dLN, what effect would this have on the Th2 response in the tissues? It is possible that the Th2 response would be amplified at the tissue site due to an increase in the number of Th2 cells there. We do not yet know what impact that would have on immunopathology or the outcome of infection. Or alternatively, a reduction in the Th2 microenvironment in the dLN may have a negative impact on the longevity of the Th2 response overall. These are all interesting questions to which we do not yet know the answers but could be addressed experimentally by a more extensive characterisation of the Th2 response in the peripheral tissues and LNs, as well as assessment of the pathology and survival of IFNAR-deficient mice, at later timepoints of infection. This is also crucial to address the timing during helminth infection when IFN-I and IFNAR signalling impacts on the immune response. Our gene expression data from *S. mansoni* infected livers suggests that IFN-I is upregulated at the induction of the dominant Th2 response (d42), but also much later during infection (d105)(Fig. 5.5). Further analysis of systemic IFN-I at different timepoints of infection will also be informative on the timing of IFN-I activity.

The suggestion that DCs may be an important source of IFN-I that influences T cell function and migration is difficult to address, as it is not yet possible to delete all IFN-I subtypes simultaneously. However, early induction of specific IFN-I subtypes – particularly IFNβ – acts in a positive feed-forward mechanism to potentiate IFN-I production via activation of IFNAR (Sato *et al.*, 1998). Thus, it has been shown that deletion of the IFNβ gene can abolish IFNα induction (Erlandsson *et al.*, 1998). It has also recently been shown that the tyrosine phosphatase PTPN22 positively regulates IFN-I production by association with TRAF3, and deletion of PTPN22 abrogates IFN-I production downstream of TLR ligation (Wang *et al.*, 2013). Thus deletion of IFNβ or PTPN22 could provide models to assess the importance of IFN-I production by DCs. However, this would require DC, or at least CD11c, -specific deletion in order to show that DC production of IFN-I is required for Th2 induction.

If IFN-I is required for optimal Th2 induction *in vivo*, this may also have therapeutic application, particularly for treatment of aberrant Th2 responses. Blockade of IFNAR could be used to limit Th2 activation in allergic individuals who suffer from asthma or food allergies. This would likely depend on first showing that IFN-I is important in the

maintenance of Th2 responses, as well as induction. However, it is likely that inhibiting IFNAR long-term would also have bystander effects on the development of immune responses against pathogens, particularly viruses. The key to this may be a very targeted management of the IFN-I response. If the specific IFN-I subtypes involved in the orchestration of the Th2 response could be identified, then it is conceivable that these IFN-I subtypes alone could be inhibited, ideally leaving anti-viral and anti-bacterial responses intact. For this to be possible, we need to identify which IFN-I subtypes are activated by Th2 Ags. A starting point for this is to use microarray analysis of FLDC subsets to identify the IFN-I genes activated following exposure to SEA. Further development of this approach would include analysis of IFN-I gene expression changes of murine BMDCs stimulated by allergy-associated Th2 Ags, such as Derp1, from house dust mites, followed by analysis of IFN-I gene expression by human DC subsets in response to Th2 Ags.

As yet we have been unable to establish whether IFN-I is active during human *S. mansoni* infection. However, given that systemic IFN-I is not easily detectable, even at the peak of viremia during virus infection, for example HBV and HCV (Stacey *et al.*, 2009), it is perhaps unlikely that a systemic IFN-I signal will be detectable in sera during helminth infection. In light of this, it is perhaps also unlikely that we would be able to detect IFN-I in the serum of those suffering from aberrant Th2 responses, such as allergic and atopic individuals. In our preliminary experiments, we have so far failed to detect IFNα secretion by human blood DCs from Western donors following exposure to SEA. Nonetheless, we remain excited by the prospect that ISGs are induced in human DCs in response to SEA. If we were able to identify an SEA-specific ISG signature from human DCs, this would open up an interesting area of research for characterisation of the IFN-I response by human DCs exposed to Th2 Ags. It would also be interesting to investigate the IFN-I signature of DCs from healthy donors exposed to Th2 or TLR Ags, and compare and contrast this response to those of DCs from helminth infected or atopic individuals – does a pre-existing Th2 environment alter the IFN-I response of human DCs?

Whilst work continues to establish the role of IFN-I in the development of Th2 responses, it is important to emphasise the value of using Flt3-L dependent BMDCs to study the recognition of helminth Ags. This method of DC differentiation adds a layer of complexity to all studies, due to the heterogeneity of the cells generated. However, this is also the

strength of this model, providing large numbers of cells for study that have clear *in vivo* counterparts. We have corroborated findings from GMDC studies that first highlighted the importance of MHC II and CD40 for the initiation of SEA-specific Th2 responses (MacDonald *et al.*, 2001; MacDonald *et al.*, 2002c)(Fig. 4.4). But we have also identified a potentially important role for IFN-I in Th2 induction. We are hopeful that further studies using FLDCs will also complement *in vivo* experiments investigating the contribution of the different DC subsets to Th2 priming in response to helminths.

7.4 Tissue-resident DC subsets in the orchestration of *S. mansoni*-specific Th2 responses

The intestines and liver are two tissues that are directly affected by egg traffic during *S. mansoni* infection, and a balanced Th2 response is essential at these sites to prevent severe morbidity and immunopathology (Brunet *et al.*, 1999; Brunet *et al.*, 1997; Fallon *et al.*, 2000; Hoffmann *et al.*, 2000). Whilst there is a growing understanding of the roles of intestinal DCs in maintenance of tolerance in the steady state (Coombes *et al.*, 2007; Jaensson *et al.*, 2008; Scott *et al.*, 2011), and the development of Th17 responses during infection with pathogenic bacteria (Persson *et al.*, 2013; Satpathy *et al.*, 2013), nothing is yet known of the role of these cells in the induction of Th2 responses during helminth infection. Characterisation of the intestinal DC subsets during *S. mansoni* infection has demonstrated that increasing egg load in the tissue altered the phenotype and make-up of the DC compartment. This included a downregulation of activation markers and a reduction in the proportion of $CD103^+$ $CD11b^+$ DCs present in the SI LP. We have also shown for the first time that $CD11c^+$ cells are required for the development of an *S. mansoni* egg-specific Th2 response in the intestine, following subserosal egg injection. These are exciting preliminary findings that suggest an important role for intestinal DCs in the development of Th2 responses against helminths.

Future experiments will address the importance of DCs in the induction of the Th2 response during patent infection, as well as extensive phenotypic and functional characterisation of the DC subsets found in the LP, intestinal lymph and MLN during *S. mansoni* infection and following egg injection into the intestinal tissue. There are a number of useful depletable mouse models that can be used in this context. Initially, we plan to

expand our preliminary data from subserosal egg injection in CD11c-DOG mice, characterising the Th2 response in the LP of these mice following egg injection, and investigating Th2 development in the MLN and LP during patent *S. mansoni* infection in this mouse model. Given that the LP MΦs also express CD11c (Schulz *et al.*, 2009), a general CD11c-depleting model is not ideal for studying the role of intestinal DCs in the induction of Th2 responses. In order to more specifically address the role of cDCs we plan to use the zDC-DTR bone marrow chimeras to selectively deplete Zbtb46-expressing cDCs (Meredith *et al.*, 2012). Use of Batf3-deficient mice will allow us to address the importance of the $CD103^+$ $CD11b^-$ DC subset in the development of Th2 responses in the intestine, whilst the pDC-DTR model (Swiecki *et al.*, 2010) can be used to rule out a role for pDCs in Th2 induction. The IRF4 (Persson *et al.*, 2013) or Notch2 (Satpathy *et al.*, 2013) DC-specific knockout mice would also be useful tools to examine the role of the $CD11b^+$ LP DC subsets in helminth-specific Th2 induction.

In the subserosal egg injection model, we have developed an ideal model with which to develop our understanding of how Flt3-L-dependent tissue-resident DCs respond to helminth Ags, in a tissue site that is highly relevant to active infection. These studies will complement our ongoing work to understand the responses of splenic DCs, which are thought to be most comparable to FLDCs (Naik *et al.*, 2005). Moreover, this model can be used to investigate the requirement for IFNAR in the optimal function of DCs and T cells in the development of egg-specific Th2 responses.

7.5 Conclusions

At the outset of the work detailed in this thesis we aimed to improve our understanding of DC subset responses to helminth Ag. In the process of pursuing this goal, we have identified an *S. mansoni* specific IFN-I signature from DCs that had been overlooked, and have begun to address the role of this pluripotent innate cytokine in the regulation of Th2 inflammation. We have also developed a relevant and exciting new model with which to study DC responses to *S. mansoni* eggs in intestinal tissues. Future work will provide a fuller understanding of the contribution of the different cDC subsets to the development and maintenance of Th2 responses, as well as elucidating the role of IFN-I in Th2 immunity.

The characterisation of helminth-specific IFN-I has the potential to revolutionise our understanding of the processes involved in the development and maintenance of Th2 immune responses, and will certainly impact on how the role of IFN-I in inflammation is viewed in future. Whilst a fuller understanding of the specific DC subsets involved in Th2 polarisation against helminths is surely very close at hand.

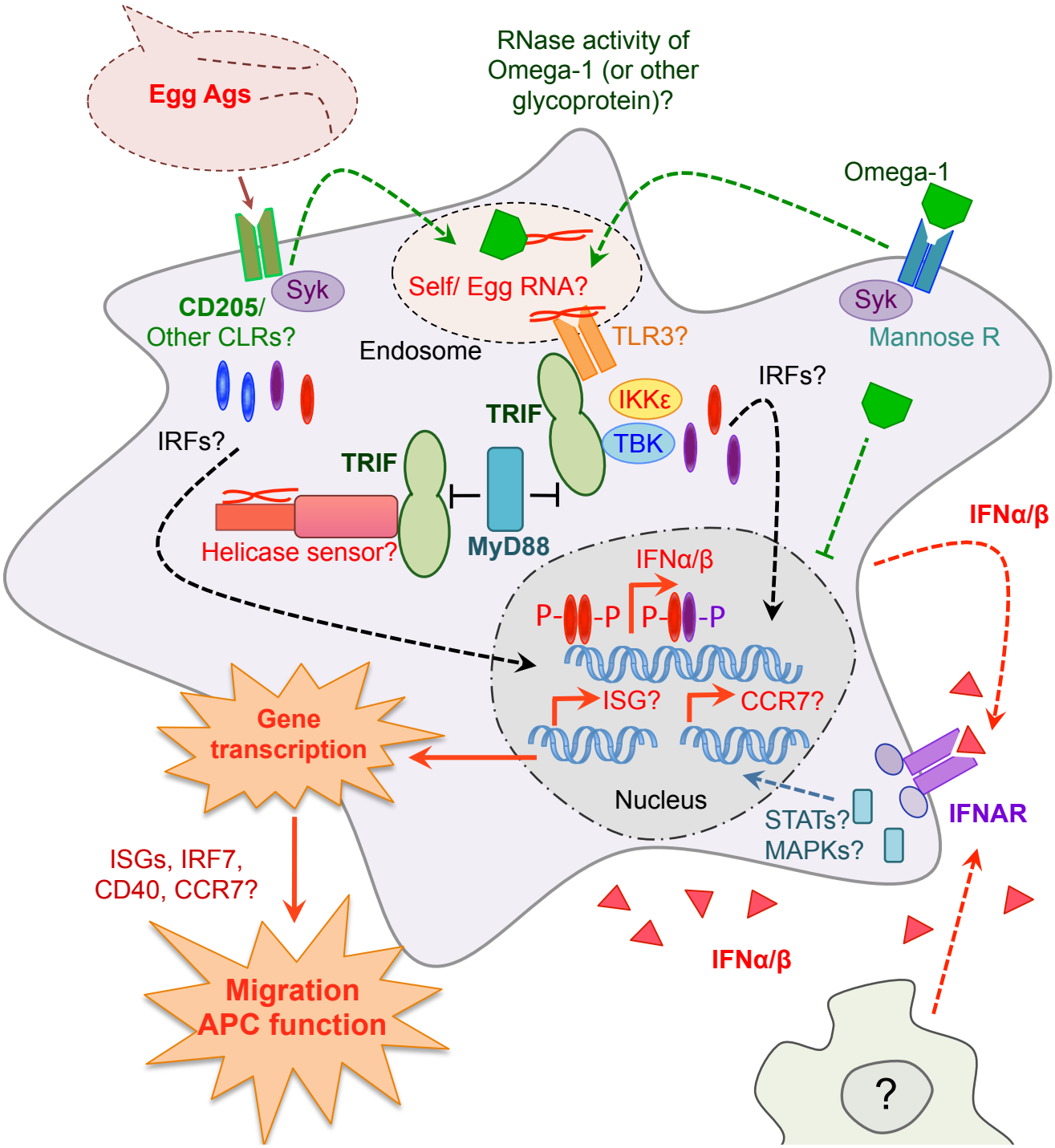

Figure 7.1 A potential positive feedback loop of SEA IFN-I in the regulation of DC function. The potential TRIF-dependent PRRs that might be involved in the induction of IFN-I include TLR3 and/ or a nucleic acid sensor. The CLR, CD205, is required for optimal IFN-I production. CD205 may activate IFN-I directly, via Syk-dependent activation of IRFs, or facilitate the internalisation of an SEA ligand capable of generating nucleic acid ligands for a TRIF-dependent PRR. Potentially, omega-1 may also generate nucleic acid ligands, or, as has been reported, inhibit protein synthesis. MyD88 negatively regulates the induction of the IFN-I response, most likely by modulating the activity of TRIF, or by inhbiting the activity of kinases involved in the activation of the IFN-I response. Binding of IFN-I to IFNAR activates the JAK kinases, TYK2 and JAK1. There are a number of signalling pathways that maybe activated downstream of IFNAR, including STATs or MAPKs. Gene expression changes induced by IFNAR signalling could enhance DC activation, migration and T cell activation.

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